ANNUAL REVIEW OF PLANT PHYSIOLOGY

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VOLUME 9

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1958

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ANNUAL REVIEWS, INC.
PALO ALTO, CALIFORNIA, U.S.A.

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PALO ALTO, CALIFORNIA, U.S.A.

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Library of Congress Catalog Card Number: A51-1660

FOREIGN AGENCY

Maruzen Company, Limited
6, Tori-Michome Nihonbashi
Tokyo

PRINTED AND BOUND IN THE UNITED STATES OF AMERICA
BY THE GEORGE BANTA COMPANY, INC.

PREFACE

The sabbatical leave is a wonderful institution. It is time for reorientation, for reflection, and for housecleaning. For a visiting American, a sabbatical leave at Oxford is particularly gratifying. To walk through the University parks; to watch soccer, rugby, and hockey on the playing fields; to follow the punts as they glide along the Cherwell; to listen to the bells at eventide; these are rare pleasures and one needs time to appreciate them. And for one interested in science, to chat with C. D. Darlington, to plan experiments with G. E. Blackman, to talk shop with W. O. James and Robert Brown, Scott Russell and E. K. Woodford, and a host of others; these are the privileges of a year at Oxford.

Oxford is venerable with age that dignifies and lends stability. And Oxford is new with the enthusiasm of youth. To hear the debate on the advisability of constructing a ten-story building on the Keble triangle that will "change the Oxford skyline," and to hear the discussion on the proposition to make Latin optional for college entrance are novel experiences for one from California.

Sitting in my pleasant living room with the sunlight streaming through the broad windows, I look out on a typical English garden. Many of the trees are one hundred years old or more; the gravel walks have been here for many generations; the sundial and the rain gauge are permanent accessories. A thing of beauty, this traditional garden has its roots in an historic past. In a California climate these trees and shrubs would have matured and declined and probably have been replaced several times in the same span of years. Such are the differences one finds in traveling from the new World to the old.

As I gaze on the dome of Radcliffe Camera and study the beauty of the Divinity School and glimpse the grizzled faces of the philosophers around the Sheldonian I am duly impressed with the antiquity of Oxford. But scanning the sky, crisscrossed with vapor trails of jet planes, I sense the spirit of unrest and change inherent in Man's nature. Sputnik, Zeta, Ghana, Indonesia, Sudan, Summit. Latin has been required for entrance at Oxford. What about science, sociology, politics? And where, in this day, stands the role of tradition?

Oxford is known for its long history of scientific progress. The Clarendon Laboratory has done much work on atomic structure; today its research in the field of low temperature is outstanding. The old school of rural economy has changed into the Department of Agriculture with emphasis on agricultural science; now it has a large group devoted to the biological application of isotopes. Here is tradition of a sound and basic sort, unswayed by the shallow popularisms of the day, confident from centuries of experience that it can meet the problems that lie ahead. Tradition, it seems to me, is of value when it stands for stability and thoroughness. But tradition for tradition's sake has no place; there is too little room for the ever-increasing mass of

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new information. Foundations we must have, but change we must have also. Change, discard, housecleaning.

We all like popular things and it is good that popularity brings intense study. But popularity fades, emphasis shifts, and new interests take over. During my years I have witnessed the ebb and flow of many emphases in the field of plant physiology. Permeability, imbibition, Donnan equilibrium, pH, auxin a and auxin b, rH, minor elements, mitogenetic rays, solarization, active water, coacervates, vernalization and photoperiod; these and many others have known the spotlight. Some are still popular, others have faded from view. Each has left its mark, many positive, some negative. And so our science has grown, becoming deeper and broader and ever more firmly planted in modern living.

Progress, it seems to me, must build on the old; make room for the new; and always keep the two in balance. Tradition has its place but so has progress. In studying the architecture of cathedral spires we must not neglect the vapor trails. While studying bound water we must save some time to consider free space. And in our fervid attempts to solve the problems of particulates we should remember the complicated procedures we have used to free them from their natural environment in the organized plant. *Annual Review of Plant Physiology* earns its place in the field of biology if it does no more than present, each year, a broad and well-balanced view of progress in this dynamic branch of science.

Last year I paid tribute to the faithful work of the Editorial Committee. Now I feel compelled to acknowledge the very great help of the assistant editors. Being permitted to edit a volume of *Annual Review of Plant Physiology* while on sabbatical leave is a rare privilege and only through the forbearance of Leonard Machlis and John Torrey was this possible. Dr. Machlis organized and chaired the annual meeting of the Editorial Committee that set up Volume 11 at Stanford. Dr. Torrey hurried home from his sabbatical leave at Cambridge to be present and help in the procedure. And Dr. Torrey is "holding the fort" in California while both Dr. Machlis and I spend sabbatical years in Europe.

The long and valuable help of Dr. James Bonner is recognized as he retires from the Editorial Committee. Dr. Anton Lang will be the new member. Mrs. Sonya Pinney worked as editorial assistant in the handling of Volumes 8 and 9; she is now succeeded by Mrs. Florene Dick; both these people have rendered invaluable service in the organization and editing of these volumes.

A. S. CRAFTS, *Editor*
Oxford, England
Spring, 1958.

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THE QUANTUM YIELD OF PHOTOSYNTHESIS[1]

By Robert Emerson

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The quantum yield of photosynthesis is derived from measurements of light intensity and rate of photosynthesis. Appropriate measurements of light intensity lead to expression of the energy absorbed by the plant material in calories per minute. The rate of photosynthesis may also be expressed in calories per minute, since it is well established that the primary product of photosynthesis is carbohydrate, and the heat of combustion of carbohydrate is known. These two primary measurements (light intensity and rate of photosynthesis) provide the basis for determining the efficiency of photosynthesis in terms of the fraction of the calories of radiation absorbed which are stored as calories of chemical potential.

Since the application of the quantum theory to photochemistry, it has been known that the elementary unit of light energy is the quantum of light, or photon. If the wavelength of this absorbed light is known, then absorbed energy, measured in calories, can be converted to number of absorbed photons. This leads to a change from the expression of percentage efficiency in terms of calories:

$$\frac{\text{number of calories stored}}{\text{number of calories absorbed}} \times 100,$$

to expression as quantum yield (quantum efficiency):

$$\frac{\text{number of molecules converted}}{\text{number of quanta absorbed}}.$$

The actual computations lead to:

$$\frac{\text{moles converted}}{\text{einsteins absorbed}},$$

the einstein representing N quanta, just as the mole represents N molecules (N = Avogadro's number, 6.02×10^{23}).

For a discussion of the origins of this application of the photochemical equivalence law to photosynthesis, and the relation between wavelength of light and energy per quantum, the reader may refer to Rabinowitch (36), chapter 29, pp. 1083–84.

If it is assumed that only a single photon can contribute to each elementary photochemical act, the energy per photon sets a limit on the chemical changes that can be brought about by a given wavelength of light, regardless of how many calories may be available for absorption. At the wavelength of the red absorption maximum of chlorophyll, the energy

[1] The survey of literature pertaining to this review was concluded in September, 1957.

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available per einstein (N photons) is only about 40 kcal. whereas the energy required for each mole of carbon converted to carbohydrate from carbon dioxide and water, and leading to evolution of oxygen, must be not less than about 120 kcal. This comparison implies that the chemical change

$$CO_2 + H_2O \rightarrow \{HCHO\} + O_2 \qquad 1.$$

should require more than one elementary photochemical act. It is believed that there are several primary photochemical steps, no one of which requires more than the energy of a single quantum of red light.

If conditions could be contrived so that every photon of light absorbed in the chloroplast would bring about a primary photochemical step in photosynthesis, and none were wasted, then dividing the number of photons absorbed by the number of carbon atoms converted to links in carbohydrate chains, would tell us how many elementary photochemical steps are involved per carbon atom. The importance of this relationship accounts for what would otherwise appear to be a disproportionately large expenditure of effort on the part of some investigators to discover conditions which would lead to the maximum observable quantum efficiency.

Furthermore, if some fraction of the absorbed photons is lost through absorption by pigments which are unable to contribute their energy of excitation to photosynthesis, this will diminish the quantum yield. The study of the quantum yield in different wavelengths of light is, therefore, a valuable approach to the question of which pigments contribute the light energy they absorb to the photochemical steps in photosynthesis.

The scope of this review is limited to a discussion of recent developments in these two fields—the maximum observable quantum efficiency, and the quantum yield as a function of wavelength of light.

There is a logical need for discussion of the maximum yield first, because although study of the dependence of yield upon wavelength might lead to significant results under conditions of less than maximal efficiency, there could always be some question as to whether the wavelength dependence would be the same under conditions of maximal efficiency.

I. The Maximum Quantum Yield of Photosynthesis

We shall use ϕ to denote the quantum yield or quantum efficiency and define it as the number of oxygen molecules produced per photon of light absorbed. So long as the organic matter produced in photosynthesis is carbohydrate (or material of the reduction level of carbohydrate), it would make no difference whether ϕ were defined in terms of oxygen evolved or carbon dioxide consumed. In either case ϕ would represent the yield per atom of carbon converted to carbohydrate according to equation 1. We use γ to denote the ratio of oxygen and carbon dioxide exchange:

$$\gamma = -CO_2(\text{moles})/+O_2\ (\text{moles}).$$

Thus, for carbohydrate synthesis, $\gamma = -1$. If the organic product of photo-

synthesis were material of a reduction level different from that of carbohydrate, γ would not be equal to -1, and the yield ϕ in terms of oxygen production would no longer represent the number of carbon atoms built into organic material. However, oxygen production might still be a good measure of the amount of energy stored in photosynthesis since the heat of combustion of organic material (per carbon atom) is to a good approximation a linear function of its reduction level [cf. Rabinowitch (36), Vol. I, p. 216]. If, on the other hand, we were to define ϕ in terms of molecules of carbon dioxide absorbed, then whenever γ was not equal to -1, there would be doubt about the amount of energy stored per photon absorbed. The quantum yield of photosynthesis is interesting especially because of the large energy requirement of the chemical changes represented, and since oxygen production is a more uniform measure of the energy storage than is carbon dioxide absorption, it is preferable to define ϕ in terms of oxygen.

As we explained in the introduction, the quantum yield ϕ must always be some fraction of unity. In many discussions of the subject, attention is centered on the number of photons absorbed per molecule of oxygen produced. This is the reciprocal of ϕ ,and must be greater than unity. It is called the quantum requirement (ϕ^{-1}). Under conditions of maximum yield, it may be hoped that ϕ^{-1} will represent the number of primary photochemical steps required for the chemical change represented by equation 1.

The principles of photochemistry lead us to expect that the energy of an absorbed photon cannot be divided among different reacting molecules, and can serve for only a single primary photochemical act. It seems inevitable that any surplus energy beyond that required for the single primary act must be dissipated as heat. As a consequence it has often been supposed that the true value of ϕ^{-1} must be an integer. Although this may prove to be the case, it is not necessarily so. Plausible mechanisms for photosynthesis leading to nonintegral values for ϕ^{-1} have been devised [cf. Rabinowitch (36), Vol. II, part 1, pp. 1138–39].

We may expect to find maximal values of ϕ (or minimum values of ϕ^{-1}) only when we come as near as possible to fulfilling the condition that each photon absorbed contributes energy to photosynthesis. There is some doubt about the extent to which energy absorbed by pigments other than chlorophyll may be contributed to photosynthesis, so most of the experiments to determine maximum yield have been made with wavelength regions in which no appreciable fraction of the incident light is absorbed by substances other than chlorophyll. (In meeting this requirement no distinction has been made between chlorophylls-*a* and *b*.) In the case of green plants, the red, orange, yellow, and green portions of the spectrum meet this requirement. Red has been preferred because chlorophyll, besides being the only cellular constituent present in sufficient concentration to absorb an appreciable amount of light, also absorbs very strongly in the red, so that small amounts of plant material suffice to absorb substantial amounts of energy. In the yellow and green regions, absorption by chlorophyll is weaker,

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so that larger amounts of cell material are required. Between the green and the blue regions, absorption by carotenoids becomes appreciable. It extends on into the blue and introduces uncertainties in the interpretation of measurements of quantum yield. Choice of the red region for the study of maximum yields implies the assumption that no important differences in yield are to be expected in different parts of the red. As we shall see in Part II, experience has shown that this is generally (though not always) true from about 580 to about 680 mμ.

Neither light absorption nor photosynthetic activity is appreciable beyond about 700 mμ. It is in the red portion of the visible spectrum that we find the longest wavelengths of light (i.e., the photons of smallest energy) capable of sustaining photosynthetic activity. Therefore, it is here also that we may expect the most efficient utilization of the absorbed energy, since with all photons of larger energy the losses must inevitably be greater. This is an additional reason why the measurements of yield in the red are of particular interest.

Books and articles dealing with photosynthesis written during the past decade, generally mention that the quantum yield is a controversial subject. Warburg, with various co-workers, has published a large number of papers in which he reports maximum yields ranging from about 0.2 to 0.36 (values of ϕ^{-1} from about 2.8 to 5). In contrast, nearly all other investigators who have measured quantum yields of photosynthesis have reported maximum values not greater than about 0.10, or at most 0.12 ($\phi^{-1} = 8$ to 10).

Much attention has been given to the theoretical implications of these different values [cf., for example, Franck (25); Rabinowitch (36), Vol. II, part 1, chap. 29; part 2, chap. 37]. The longest wavelengths of red light which sustain full photosynthetic activity provide about 40 kcal. per einstein. The change in free energy represented by equation 1 is approximately 120 kcal. If we could imagine that all the energy of each absorbed photon might be applied to photosynthesis with no heat losses whatever, then 2.8 would be the smallest number of photons which could conceivably suffice per carbon atom converted to carbohydrate. For longer wavelengths of red light, this would represent an efficiency of about 100 per cent. Warburg interprets his results as proving that green plant cells (*Chlorella*, to be specific) can indeed attain such efficiencies. Rabinowitch, Franck, and others who are familiar with the principles of photochemistry and chemical kinetics, have said that it is not conceivable that the excitation energy resulting from absorption of light could bring about photosynthesis entirely without heat losses. Franck has made careful estimates of what these losses might amount to (25, pp. 198–99). He concludes that neither three nor four photons of red light could conceivably meet the energy requirements per atom of carbon converted according to equation 1. In his opinion the smallest number that could possibly suffice would be five or six, and with eight to ten photons there would be a reasonable margin for heat losses.

According to these considerations, the quantum yields of 0.10 to 0.12,

reported from most laboratories where the subject has been studied, appear to represent the maximum that could be expected, while the yields of .20 to .36 reported by Warburg are so high that they seem quite irreconcilable with theoretical expectations.

Franck (25) has offered plausible suggestions to account for the remarkably high yields reported by Warburg, as well as for the lower maximum values found by other observers. However, before we give further attention to theory and interpretation, it is altogether proper that we look first at the experimental evidence supporting the divergent values at hand. If either the higher or the lower values reported for the maximum yield should prove to be founded upon experimental work of questionable reliability, there might be no need to search for an interpretation which would account for both ranges of values.

Errors in the measurement of the energy of the incident light beam, or in the estimation of the fraction of the incident beam absorbed by the cells, or in the measurement of the rate of photosynthesis are possible, and one might anticipate that differences in the plant material could account for differences in yield.

There is no evidence whatever that the differences in yield are to any significant extent attributable to errors in measurement of incident light intensity. The techniques for measurement of radiation are well known, and the comparisons made between the methods and results of different laboratories have not suggested that the large discrepancy between the higher and lower yields could be accounted for by differences in radiation standards or in the application of techniques for measuring incident intensity.

On the other hand, the measurement of the scattered light transmitted by cell suspensions (upon which depends the estimate of the fraction of the incident beam which is absorbed) requires techniques which are not standardized and which might easily lead to significant errors. We shall be obliged to consider these techniques in connection with the measurement of quantum yields with thin suspensions.

Regarding the plant material there are, of course, ample opportunities for large physiological differences, even though most of the work on quantum yields has been done with a single species (*Chlorella pyrenoidosa*), and though the strains of this organism in use in various laboratories show remarkable physiological uniformity. It might be supposed that Warburg, alone of all the investigators who have worked on this problem, has developed the technique of culturing cells capable of maximum efficiency of photosynthesis. Several considerations make this seem unlikely. In the first place, Warburg's specifications for producing cells of highest efficiency, though often stated with emphasis upon their importance, show no consistency. Often the specifications are contradictory. He has reported high quantum yields for cells grown under a wide variety of conditions. Comparison of his specifications with those followed by others who have obtained lower yields suggests no significant differences, and there is every reason to

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conclude that no particularly narrow range of culture conditions must be adhered to in order to produce cells capable of giving the results which Warburg reports. The rates of growth observed in different laboratories are similar, suggesting that culture conditions have generally been favorable, but this in itself may be of no great importance, since Warburg has found high efficiencies with both slow-growing and fast-growing cells.

On the other hand, there is every reason for regarding the measurements of rate of photosynthesis as the most probable source of the difference between the higher and lower values for maximum quantum yield. The rate of photosynthesis is generally derived from two measurements, one for respiration, and one for the sum of respiration and photosynthesis. But there is a wide possibility for choice of light and dark intervals from which to derive these rates, and the procedures Warburg has devised for measurement of quantum yields can lead to very different rates of photosynthesis depending upon the time intervals chosen.

Respiration may become of negligible importance if measurements are made with optically thin suspensions, and some of Warburg's high yields are derived from such measurements. Here there should be no problem of choice of time intervals, but as we shall see, there has nevertheless been a selection of intervals. Also the magnitude of possible error in the estimation of the fraction of light absorbed becomes much larger than in measurements with suspensions of greater optical density.

With one exception, all of Warburg's measurements of rate of photosynthesis were made manometrically, and most of them were by the so-called two-vessel method. In contrast, investigators seeking to verify Warburg's claims have used a wide variety of different methods, including manometry [Emerson & Lewis (20)], gas analysis [Manning *et al.* (32)], calorimetry [Arnold (1)], polarography [Brackett *et al.* (4)], the dropping mercury electrode [Moore & Duggar (33)], and a combination of infrared spectroscopy for carbon dioxide determination and paramagnetic measurements for oxygen determination [Yuan *et al.* (55)]. Since none of these other methods has led to quantum yields consistently higher than about 0.12 (and most have approached this value as a limit), and since manometry, in laboratories other than Warburg's, has failed to confirm his results, the problem seems to narrow down to whether Warburg's special applications of manometry may be the origin of the large difference between his yields and those reported by others.

The one case in which Warburg appears to have found a high yield by a nonmanometric method is the work of Damaschke *et al.* (9). Here a polarographic method was used, and the results were said to be in agreement with those from manometric measurements. In this publication Figure 6 shows large inconsistencies between the slopes of the lines and the rates marked beside them. Because of these inconsistencies, it is impossible to be sure what has been the basis for computation of the quantum yields. This publication falls short of providing clear evidence that a nonmanometric method can lead to quantum yields of 0.25 or higher.

Warburg's manometric experiments remain his strongest evidence of high quantum yields. We are confronted with a considerable number of papers describing many different types of measurements under a wide range of conditions. Rabinowitch (37, pp. 370–74) has reviewed the papers up to 1951. At that time Warburg was making measurements with optically dense suspensions, mostly by two-vessel manometry. The limitations which enter into the application of this technique to the measurement of steady rates have been reviewed by Nishimura *et al.* (34), by Emerson & Nishimura (23), and by Pirson *et al.* (35). Warburg's application of the technique has generally been such as to include in the rate measurements periods during which the rates were undergoing changes (from dark rate to light rate, and vice versa). Emerson & Chalmers (16) have shown that application of the two-vessel technique to periods of transient changes in rates is subject to additional limitations beyond those which apply to the measurement of steady rates. Warburg applied the technique without regard to these limitations. He and his co-workers did not observe evidence of transient changes in rates, and repeatedly expressed the opinion that in their experiments the manometric readings responded without observable periods of transition, to the changes in rate brought about by the sequence of light and dark periods [for example, Warburg *et al.* (44, p. 337)]. Emerson & Chalmers were able to offer a plausible explanation for the failure of Warburg and co-workers to notice evidence of transient changes in response of the manometers. The sudden and brief bursts of pressure change characteristic of *Chlorella* metabolism during the first minutes of light and dark periods could conceivably make it appear that the manometric readings come instantaneously to the new rates of pressure change. These pressure bursts are generally due to extreme changes in the rate of carbon dioxide exchange. Mass spectrography [Brown & Whittingham (7)] has confirmed these fluctuations in rate of carbon dioxide exchange under the general conditions of Warburg's experiments, so there is every reason to believe that they must have been present in Warburg's experiments, too, and might have misled him to the belief that the manometric responses were instantaneous. Such a conclusion cannot be sustained by the experimental evidence.

Emerson & Chalmers modified the technique of two-vessel manometry for the special purpose of studying transient changes in rates of gas exchange and measuring quantum yields according to Warburg's specifications. For the most part their experiments confirmed the conclusion reached earlier by Emerson & Lewis (20) and later confirmed by Brown & Whittingham (7) that the pressure bursts were attributable to carbon dioxide exchange. They reported occasional evidence that under some conditions oxygen as well as carbon dioxide might contribute to the pressure bursts. They did not regard the evidence as conclusive, but they showed that if taken at face value it could lead to quantum yields for oxygen production as high as those reported by Warburg. This was only possible if the highest rates of oxygen absorption in darkness were assumed to be representative of the rates of respiration during the moments of maximum indicated oxygen production

in the light, an assumption which seemed to them improbable although it appears to be the foundation of Warburg's high yields in so far as they are derived from experiments of the same type. Emerson & Chalmers regarded the very high rates that could be calculated from such measurements as speculative, and suggested that even if they were real, their transient character made it more likely that they represented oxygen exchanged from interconversion of metabolic intermediates, rather than from carbohydrate synthesis according to equation 1. Oxygen exchanged from pools of metabolic intermediates would represent energy changes of unknown magnitude, but they would probably be small compared to the energy changes in photosynthesis. Thus, the quantum yield of oxygen production derived from measurements of transient rates would be of doubtful significance for the problem of how many photons are required for photosynthesis.

From the steady rates of oxygen exchange indicated by the experiments of Emerson & Chalmers, quantum yields of 0.10 to 0.12 could be calculated, in agreement with the results obtained in many other investigations.

Steady rates, exclusive of periods of transient changes immediately following light-dark and dark-light changes [or addition of increments of light to continuous background illumination, a procedure sometimes used by Warburg (46, pp. 419–21)] seem to be a more reliable basis for measuring quantum yields than transient rates which represent chemical changes of uncertain energy requirement. Without making direct answer to the questions raised about his earlier techniques, Warburg made new measurements with optically thin suspensions, so thin that the rate of respiration became negligible in comparison with the rate of photosynthesis, and derived quantum yields from experiments in which the cells were in continuous light of constant intensity for periods of hours (49). The rates must, therefore, be considered free from errors associated with the application of the two-vessel method to the measurement of transient rates, but for measurement of steady rates equality of light absorption in the two vessels remains an indispensable requirement. Equality must be maintained during the conditions of shaking which prevail during the measurements. Warburg mentions adjusting the two light beams to equality of intensity, but this does not assure equality of absorption in the two vessels, even though they contain equal quantities of cell material suspended in equal volumes of fluid. In Warburg's application of the two-vessel method, the two vessels are of different shape, a feature of his technique which was discussed by Emerson & Chalmers with special reference to its effect on measurement of transient rates. In the case of steady-rate measurements with optically thin suspensions, a pair of vessels such as Warburg's cannot be counted upon to absorb equally, since the average distribution of the cell suspension is more toward the ends of the high-sided vessel, allowing for greater transmission of light than in the low vessel. Even with vessels of the same shape, equality of intensity of the incident beams does not assure equality of light absorption, because different vessels scatter, reflect, and refract differently [cf. Emerson & Chalmers

(16, p. 510)]. It would have been fortuitous if Warburg's adjustment of the two beams to give equal incident intensity had provided equality of absorption in the two vessels. The difference to be anticipated because of difference in shape (greater transmission in the case of the deep vessel) would lead to too great a difference between the pressure changes in the two vessels, and hence to over-estimation of the rate of photosynthesis. Reference to Pirson's (35) analysis of the application of the two-vessel method to measurement of steady rates shows that a small inequality between the rates in the two vessels leads to a disproportionately large error in the calculated rate of photosynthesis.

Besides the uncertainty about equality of absorption in the two vessels, there is also uncertainty about the fraction of the incident light which was absorbed by the cells. Warburg derived this from measurements of transmitted light, made with an Ulbricht sphere. His specifications for use of this device (like many of his specifications in regard to details of experimental technique) differ greatly from one instance to another, so generalization is impossible. In some cases (49, p. 679) he derived absorption from transmission values indicated by the sphere for cell suspensions and for corresponding samples of clear suspending fluid. Since the incident beam must have been more strongly scattered by the cell suspension than by the clear suspending fluid, there must have been large differences in the pattern of distribution of scattered light inside the sphere. To those who have applied the Ulbricht sphere to measurements of absorption by scattering media, it is well known that the energy indicated by a light-sensitive element located at a fixed point in the sphere varies with the pattern of distribution of scattered light. To minimize errors in estimation of absorption, it is important to use as a nonabsorbing control some medium which gives a scatter pattern approximating that of the absorbing sample [cf. Kok (29, pp. 5–16)]. In a later paper Warburg & Krippahl (48) prescribed different steps for application of the Ulbricht sphere, which included a measurement with a suspension of white cells from which the pigments had been extracted with methanol. They mentioned no tests of scatter pattern, though again it is well known that extraction of the chloroplast pigments does not leave the scattering properties of the cells unaltered.

Whatever may be the uncertainty in the measurements of the transmitted light, the uncertainty in the derived value for the absorbed energy is bound to be larger if the absorption is a small fraction of the transmission. For example, if there is an uncertainty of ± 2 per cent in the estimation of transmitted light, and if about 90 per cent of the incident energy is transmitted, then the uncertainty in the absorbed energy is ± 20 per cent. Warburg reports quantum yield measurements with suspensions which absorbed as little as 5 per cent of the incident light. He did not derive such small absorption values from direct measurements of transmission, but obtained them indirectly by linear extrapolation from direct measurements with suspensions two and four times as dense (or sometimes three and six

times as dense). Warburg's own estimates of the precision of his technique (48, p. 182) seem much too optimistic, and the cruder techniques described in connection with some of the quantum yield measurements [for example, Warburg *et al.* (49)] leave room for an uncertainty of ± 20 per cent in the estimates of light absorption.

The manometric measurements with thin suspensions also show evidence of considerable uncertainty. For example, we may consider Fig. 1 in the paper by Warburg *et al.* (49). The figure shows pressure changes plotted against time, for a certain pair of manometer vessels. Both appear to have maintained nearly steady rates of pressure change for 5 hr., and from the appearance of these curves the reader might expect that there would be no significant difference in rate of photosynthesis, regardless of what portion of the 5 hr. were used for calculation. The figure shows an arrow at the 1-hr. point, indicating that at this point a value of $\phi^{-1} = 4.3$ was calculated. The rate of photosynthesis indicated by two-vessel measurements depends upon the ratio H′/H. The data for Fig. 1 (tabulated in Section 16, Expt. 2) show that in the course of 5 hr., different pairs of values gave ratios varying from a minimum of 1.78 (the period from 120 to 180 min.) to a maximum of 2.23 (the first 30 min.). The ratio for the entire period is 1.85. This ratio determines the factor by which the rate of pressure change H′ is to be multiplied to give the indicated rate of oxygen production. For the vessels and liquid volumes specified in this paper, a ratio of 1.78 gives a factor of 2.16, and a ratio of 2.23 gives a factor of 3.24. This means that the highest rate of photosynthesis that could be calculated from pairs of pressure measurements in this experiment is 50 per cent higher than the lowest rate. At the point indicated by the arrow in Fig. 1. the ratio 2.02 indicates a factor of 2.80, 16 per cent higher than the factor 2.41, which would be the average for the entire 5-hr. period of this experiment.

All the experiments tabulated in this paper show similar large variations in the ratio H′/H. For experiment 1 the range is from 1.78 to 2.18; for experiment 3 it is from 1.74 to 2.25; for experiment 4 it is from 1.89 to 2.00. This corresponds to an uncertainty in the rate of photosynthesis of ± 25 per cent, which hardly justifies the precision with which values of ϕ^{-1} have been expressed. It may be quite accidental that the times indicated by arrows in Fig. 1 and 2 lead to nearly equal yields, but the fact remains that the calculation of rates from other time intervals in these experiments would have led to widely divergent values.

There is room for difference of opinion on the interpretation to be given to the facts as stated here. Conceding that among the manometric measurements there is evidence of rates of oxygen production which indicate quantum yields of 0.25 or higher, one could then seek plausible interpretations of these yields, which seem too high to be representative of photosynthesis according to equation 1. On the other hand, one could say that because of certain weaknesses in the experimental techniques and the uncertainty of some of the assumptions on which these high rates of oxygen production

have been calculated, and also because, by alternative treatment of original data from the same experiments, much lower rates can equally well be calculated, therefore, the exceptionally high rates should be regarded as spurious, and there would be no need for interpretation. The reviewer is of the opinion that this latter viewpoint is the one to be preferred on the basis of evidence now at hand. It seems not unreasonable to say that if we seek to decide what is the most probable value for an important biological constant, such as the quantum yield of photosynthesis, we should feel free to omit from consideration such experiments as appear to be subject to large errors because of evidence of faulty application of technique or unsupported assumptions.

Franck (25) has explored the alternative of accepting the reported yields as truly representative of oxygen production, and has offered a plausible interpretation. His suggestion is that metabolic intermediates more reduced than carbon dioxide may enter the chain of photosynthetic reactions and lead to oxygen production at smaller energy cost, and therefore at higher quantum yield, than could be possible for photosynthesis starting from carbon dioxide and water.

Evidence from various sources supports the hypothesis that intermediates from respiration may enter the chain of reactions of photosynthesis and it may prove to be correct regardless of whether it is the true explanation for Warburg's quantum yields. For example, Kok (28) has found a sharp difference in yield of photosynthesis above and below the compensation point. Below compensation, where respiration could supply intermediates at a rate greater than the rate of photosynthesis, Kok reports a yield about twice as great as above compensation, where the rate of respiration would be insufficient to sustain oxygen production entirely from intermediates, and carbon dioxide would presumably have to be drawn into the process. The sharp breaks shown at the compensation point in Kok's figures imply that immediately above compensation, carbon dioxide should completely replace the metabolic intermediates. A gradual change would seem more plausible. Careful search by other investigators has sometimes failed to show the "Kok effect." Emerson & Lewis (22, Fig. 1) observed no sharp change at the compensation point, and found nearly the same yield both above and below. Emerson & Chalmers (17) measured the yield of photosynthesis for cells with widely differing rates of respiration, so that the photosynthesis for a given light intensity constituted either a small fraction of the respiration or a net gain in oxygen production in excess of respiration. The yield was the same in both cases.

Van der Veen (41, p. 227) mentions observation of the "Kok effect," but has not confirmed it in later experiments. Bassham *et al.* (3, p. 336) show a figure indicating a change in yield at low light intensities, but no measurements were made below the compensation point. The evidence of change in yield above compensation is hardly conclusive. The direct observations of photosynthesis as a function of light intensity, uncorrected

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for respiration, lie on a straight line, and a small difference in the corrections applied for respiration could eliminate the slight departure from linearity shown for the corrected values. The authors assumed that the rates of respiration following the light exposures were correctly representative of the rates during light exposures. The more usual practice is to average the rates of respiration before and after light exposures, and the fact that in these experiments the rate of respiration increased with increasing light intensity is evidence that an average from readings before and after light exposures would be more nearly representative. The tabulated data suggest that such a change would bring the corrected rates of photosynthesis to lie close to a straight line, the slope of which would represent a quantum requirement of about 7. These experiments do not appear to provide supporting evidence for the "Kok effect," or for higher quantum yields below compensation of respiration.

The small amount of positive evidence for the "Kok effect," together with negative results in several instances of search for confirmatory evidence, leads to the conclusion that a substantial difference in quantum yield above and below the compensation point is not a general phenomenon. If it is something associated with special conditions, these conditions have yet to be specified. It seems equally possible that the apparent positive evidence arises from changes in rate of respiration between the dark and light intervals chosen for calculation of rate of photosynthesis. In all measurements of photosynthesis at or near the compensation point, any changes in rate of respiration between light and dark periods lead to corresponding errors in the calculated rate of photosynthesis. In the reviewer's experience, the rate of respiration during a series of exposures to light of different intensities is never constant, and distortion of the curve for rate of photosynthesis as a function of intensity can be avoided only by checking the rate of respiration before and after exposure to each light intensity.

If the various weaknesses of Warburg's experimental evidence for high quantum yields are overlooked, his results as they stand offer little in support of Franck's suggestion that the high yields are due to substitution of intermediates from respiratory metabolism for carbon dioxide. Franck offered his explanation at a time when Warburg's evidence of high yields was derived from measurements with dense cell suspensions in which the average light intensity was near compensation of respiration, but there was no evidence of a difference in yield above and below compensation, and high yields had also been obtained by adding a small increment of measured light to a background illumination of intensity sufficient to maintain average photosynthesis well above compensation (43). In Warburg's later measurements with optically thin suspensions, respiration was a negligible fraction of photosynthesis, so it could not be supposed that intermediates of respiration were being produced at a rate sufficient to sustain an appreciable fraction of the observed rate of photosynthesis. Warburg & Krippahl (47, p. 495) did not accept Franck's suggestion, and regarded their yields as

representative of complete photosynthesis according to equation 1.

Although Warburg did not think that the reduction of intermediates from respiration made any significant contribution to the high quantum yields which he observed, he did report evidence of a contribution of energy derived from oxidative metabolism to the process of photosynthesis (8, 46). This evidence was derived from exceptionally short periods of exposure to the beam of measured light (1 to 3 min.), alternated with short dark periods or periods without the measured light. For these short periods, he observed exceptionally high rates of pressure change, which he interpreted as showing that during the first moments of illumination, oxygen was produced with a quantum yield of unity. Warburg's account of these measurements has given some readers the impression that he has demonstrated a quantum yield of unity for photosynthesis. Such a yield would, of course, be in contradiction to the law of conservation of energy; Warburg makes no such claim for his results. His interpretation (see especially 45, 51) is that the difference between the energy requirement for photosynthesis and the energy available per quantum of light can be made up, under the special circumstances of these experiments, by energy derived from oxidation of previously synthesized carbohydrates. He says that in rapid alternations of short periods of light and darkness he can detect, in the first moments of darkness, the exceptionally high rate of respiration which sustained the quantum yield of unity for oxygen production during the short light period.

Any oxidative metabolism such as is assumed here could only be sustained at the expense of carbohydrates previously synthesized. Warburg says that two-thirds of the carbohydrate synthesized with a quantum yield of unity is reoxidized, so that the quantum yield for the net gain in carbohydrate production is only about 0.3, in agreement with what he finds from his other experiments already discussed.

These interpretations offered by Warburg imply that the rate of respiration during illumination must have greatly exceeded the steady rate of respiration measurable in darkness. The rate of respiration during photosynthesis cannot be directly measured by classical methods, but Brown (6) devised a technique for direct observation of rate of respiration during photosynthesis, based on mass spectrography and enrichment of the air above a suspension of cells with the heavy isotope of oxygen ^{18}O. Illumination resulted in no appreciable effect on the rate of respiration of *Chlorella* when the algae were adapted to the light intensity used. This was also the case for certain other algae and vascular plants tested. In certain experiments with algal cells there were changes in isotope ratio which might have been interpreted as evidence of differences in rate of respiration in light and darkness, but Brown thought it more plausible to attribute these changes to direct reutilization of the oxygen produced in photosynthesis, rather than to an effect of light on rate of respiration.

The measurements which Warburg interprets as evidence for a quantum yield of unity were made by the two-vessel method, and rates were

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calculated without any allowance for time lag in the response of the manometers. All the criticisms made with respect to Warburg's other manometric measurements of quantum yields are applicable to these measurements also, and with even greater force. For one who is familiar with the limitations of the two-vessel method, it is difficult to understand how these observations could be regarded as adequate to establish the conclusions drawn, or indeed to give any quantitative information whatever.

Even though the experimental evidence in support of Warburg's claims for quantum yields from 0.25 to 0.36 must be regarded as inadequate, this does not establish the yields of 0.10 to 0.12 as the maximum attainable. Among the data published by various authors (and generally interpreted as indicating a maximum yield of about 0.12) are occasional values intermediate between those reported by Warburg and those generally mentioned by others as the maximum that can be sustained by their experimental evidence. For example, Brackett *et al.* (4) mention a lower limit of 6 for their quantum requirements, and a mean of 8.5. Yuan, Evans & Daniels (55, p. 189) tabulate several values of ϕ^{-1} between 6 and 8. Therefore, it cannot be said that 0.12 represents the maximum yield observed by investigators other than Warburg and his co-workers.

Daniels (55) regards the values between 6 and 8 obtained in his laboratory as within the normal range of scatter to be expected in any large number of measurements of this kind, and considers the evidence for a minimum value of 8 for ϕ^{-1} ($\phi = 0.12$) as reasonably conclusive. Brackett and his co-workers, on the other hand, think their values of ϕ^{-1} below 8 are statistically significant.

The value of 4 for ϕ^{-1}, first proposed by Warburg as the probable minimum in 1923, became especially attractive when it was realized that the reduction of carbon dioxide to carbohydrate requires the transfer of four hydrogen atoms. Franck has made it appear unlikely that the energy of four photons of red light would suffice to cover the changes in free energy, plus the inevitable losses. If one photon per hydrogen atom is insufficient, it is easily conceivable that each hydrogen atom could be moved in two steps, each step requiring one photon. Four hydrogens moved in two steps each would require a minimum of 8 photons. Undoubtedly this has inclined people to regard values in the neighborhood of 8 as evidence that 8 is the true minimum value.

If we could be sure that ϕ^{-1} were some integer, the various measurements which enter into the determination of the quantum yield of photosynthesis are sufficiently precise to enable us to distinguish between 3, 4, and 5. With techniques available today, they are not sufficiently precise to distinguish between 7, 8, and 9. The combination of the experimental evidence and the theoretical reasons for expecting that ϕ^{-1} will be some multiple of 4, appear to justify the provisional conclusion that 8 is the probable minimum value, but the experimental evidence alone would only justify 8 ± 1, and we cannot yet be sure that the minimum possible value of ϕ^{-1} must be an integer.

II. The Quantum Yield as a Function of Wavelength, and the Yield for Light Absorbed by Pigments Other than Chlorophyll

Direct quantitative evidence that pigments other than chlorophyll can contribute their excitation energy to photosynthesis was found by Emerson & Lewis (21) for phycocyanin, by Dutton & Manning (10) for fucoxanthol, and by Haxo & Blinks for phycoerythrin (27). As explicitly stated by Emerson & Lewis (21, pp. 590–91), the reasoning by which this conclusion is reached includes the assumption that the quantum yield for light absorbed by chlorophyll is the same throughout the spectral range of its activity in photosynthesis. Chlorophyll is thus the yardstick against which the yield for other pigments is measured.

It seemed unlikely that pigments so diverse chemically as the phycobilins, the carotenoids, and the chlorophylls, could all be directly linked to the enzymatic reactions which ensue from the absorption of light. Therefore, from the first it seemed probable that excitation energy must be transferrable among the absorbing molecules. Arnold & Oppenheimer (2) calculated the probability of transfer from phycocyanin to chlorophyll, and concluded that it might take place with high efficiency. Dutton *et al.* (11) observed chlorophyll fluorescence in live cells when practically all the exciting light must have been absorbed by fucoxanthol, showing clearly that there must have been transfer of excitation energy from fucoxanthol to chlorophyll. French & Young (26) reported similar evidence of transfer from phycoerythrin to phycocyanin and to chlorophyll. Duysens (12, 13) made a more comprehensive study of energy transfer among all the chloroplast pigments. The significance of these several investigations has been discussed by Rabinowitch (36, pp. 1299–1310, 1868–79; 38, pp. 238–44).

These various lines of evidence concerning energy transfer have led to a more concrete picture of the manner in which the different pigments may contribute their energy of excitation to photosynthesis. Chlorophyll-*a* is now regarded as the pigment directly responsible for utilizing the excitation energy for the enzymatic steps in photosynthesis. Chlorophyll-*a* may receive its excitation by direct absorption of light, or by transfer of excitation from some other pigment. The path of transfer is from those pigments with absorption bands at shorter wavelengths, to those with bands at longer wavelengths. The overlapping of the fluorescence band of the donor pigment with the absorption band of the accepting pigment increases the probability of transfer. There is also evidence of energy transfer among like molecules [Lavorel (30)]. Of all the pigments associated with the organs of photosynthesis, chlorophyll-*a* shows absorption extending farthest toward long wavelengths, and shows an absorption maximum at a longer wavelength than that of the longest-wave maxima of any of the other pigments, excepting only chlorophyll-*d*. This means that the energy level of the first excited state of chlorophyll-*a* is lower than that of any of the other pigments (except chlorophyll-*d*). Therefore, all absorptions of shorter wavelengths, by whatever pigment, have a good chance of leading to the first excited singlet state

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of chlorophyll-*a*. If primary absorption were by fucoxanthol, excitation would pass through chlorophyll-*c* to chlorophyll-*a*. If primary absorption were by some carotenoid of green algae, the path might be through chlorophyll-*b* to *a*. Evidence gathered by Duysens indicated that these transfers could take place with very high efficiency.

This evidence that energy absorbed by all the various pigments leads directly or indirectly to excitation of chlorophyll-*a*, and hence that chlorophyll-*a* must be the direct sensitizer for photosynthesis, leads to a slightly different viewpoint on the interpretation of measurements of the quantum yield of photosynthesis as a function of wavelength of light. The yield for light absorbed by any particular pigment should depend upon the efficiency with which its excitation energy can be transferred to chlorophyll-*a*. This may not be the same under all circumstances. It may depend upon the relative concentrations of the two pigments, and on the arrangement of the pigment molecules in the chloroplasts or grana, as well as on the degree of overlap in the bands of fluorescence and absorption. Under the circumstances, one should be prepared to find that the yield for light absorbed by any particular pigment may not always be the same.

This concept (which we owe largely to Duysens) of the migration of excitation energy from all the chloroplast pigments toward chlorophyll-*a* has offered a satisfactory explanation for the results obtained with most types of algae, but in the case of red algae some additional assumptions were required to account for the dependence of photosynthesis upon wavelength. Haxo & Blinks (27) reported that their measurements of action spectra indicated a lower yield for light absorbed by chlorophyll than for light absorbed by phycoerythrin. This was confirmed by absolute measurements of quantum yield by Yocum & Blinks (54). Duysens found a similar relationship for the yield of chlorophyll fluorescence. Haxo & Blinks had suggested that in red algae, phycoerythrin might play a more important part than chlorophyll. Duysens retained the hypothesis that chlorophyll-*a* is the means by which excitation energy is made available for photosynthesis, but proposed that in red algae chlorophyll-*d* might act as a terminal sink for the excitation energy, thus drawing the energy away from chlorophyll-*a*. Rabinowitch (38, p. 239) discussed possible reasons why the energy received by chlorophyll-*d* might be unavailable for photosynthesis. In order to account for the higher yield of both photosynthesis and the fluorescence of chlorophyll-*a* when primary light absorption is by phycoerythrin than when chlorophyll-*a* absorbs directly, Duysens suggested that the chlorophyll-*a* is present in two forms, one of which is efficient in fluorescence and photosynthesis, and the other of which is inactive, presumably because it passes its excitation energy along to chlorophyll-*d*. Duysens supposed that energy first absorbed by phycoerythrin is transferred preferentially to those chlorophyll molecules which are efficient in photosynthesis.

It is not yet clear whether it will remain necessary to resort to special assumptions to explain the dependence of yield of photosynthesis upon wave-

length in the case of red algae, or whether a common interpretation covering algae with all combinations of pigments can be devised. The need for special assumptions arises from experimental evidence which is not altogether conclusive, and the results of some recent experiments suggest alternative possibilities.

Haxo & Blinks (27), as well as Yocum, relied upon integrating devices for measuring the light transmitted by samples of algal thallus. Yocum's integrating sphere was an improvement over the integrating hemisphere described by Haxo & Blinks. Both methods probably lead to a close approximation for the absorbed light, when absorption is large compared to scattering, but Yocum (53, pp. 38–39) emphasized that in spectral regions where absorption is small, the integrating devices become less precise. We may conclude that where absorption is low, the estimated quantum yields are less certain, so the exact shapes of action spectra are also uncertain. In the case of red algae, absorption is small in the red part of the spectrum; it is in this region that the precise dependence of yield upon wavelength is doubtful. Especially in the red-orange region, where phycocyanin and chlorophyll are both contributing to absorption, it is hardly possible to be sure of the fraction absorbed by chlorophyll, or the yield to be assigned to it, although there can be no doubt that farther out in the red, where absorption is due to chlorophyll alone, the yield becomes very low.

The experiments of Marcia Brody (5) add important new evidence because they were made with optically dense suspensions, which gave total absorption of the incident light, thus avoiding uncertainty about the fraction absorbed. With the red cadmium line (644 mμ) she found a yield of about 0.10, in agreement with, or generally a little higher than, the yield at 546 mμ, where phycoerythrin is the principal absorbing pigment. At 644 mμ, absorption is divided between chlorophyll-*a* and phycocyanin. *Porphyridium* cells contain only a small amount of phycocyanin, so at 644 mμ at least half of the total absorbed energy must be absorbed by chlorphyll. Therefore, Brody's results show that unless the light absorbed by phycocyanin is much more efficient than the light absorbed by phycoerythrin, the fraction absorbed by chlorophyll must be active at near-normal efficiency. Her measurements at 644 mμ do not support the hypothesis of Haxo and Blinks that chlorophyll as a whole is less active in photosynthesis than phycoerythrin, nor the hypothesis of Duysens that some substantial portion of the chlorophyll is inactive.

Beyond 644 mμ, in the region of the absorption maximum of chlorophyll-*a* Brody generally found low yields with totally absorbing suspensions, in agreement with the evidence of low yields found by Haxo & Blinks, and by Yocum, with optically thin material. Brody suggested that this region of the spectrum was beyond the range of maximum yield, and that the decline in longer wavelengths of red was analogous to that observed by Emerson & Lewis (22, Fig. 5, 6) for *Chlorella* photosynthesis beyond 680 mμ. In both *Chlorella* and *Porphyridium* the decline begins within the red absorption

band of chlorophyll-*a*, but in the case of *Porphyridium*, it begins on the short wavelength side of the maximum.

Brody has found that the location of the long-wave decline is not constant for *Porphyridium*, but can be shifted by exposing the cells to different colors of light. Exposure to green light for a period of hours resulted in extending the region of the decline to coincide more or less with the position of the decline found by Emerson & Lewis for *Chlorella*. Haxo & Blinks had reported that exposure of red algae to red light for periods of days increased the yield in red, again perhaps evidence of change of the long-wave limit rather than of general change in the efficiency of chlorophyll.

Emerson and co-workers (18, 19) have further investigated the long-wave limit for photosynthesis of *Chlorella* and compared the results with those of Brody for *Porphyridium*. In each case they found that lower temperatures shifted the decline toward longer wavelengths. They also observed that in the region of the long-wave decline, supplementary light of shorter wavelengths could bring the yield for the long-wave red up to the level of the yield for shorter wavelengths of red, i.e., up to the level of maximum yield. (These effects of supplementary light seem to be fundamentally different from those described by Warburg *et al.* (50, 52), since Warburg characterizes his effects as obtainable with intensities of blue light too small to give appreciable photosynthesis, while Emerson and co-workers found it necessary to use intensities of supplementary light high enough to give measurable photosynthesis.) In a brief note (15), Emerson mentions that they have studied the effect of supplementary light of different wavelengths and intensities and found that for *Chlorella* the wavelengths most effective in raising the low yield for long-wave red (i.e., the wave-lengths giving equivalent effect at lowest intensities), are in spectral regions where absorption by chlorophyll-*b* is strong compared to absorption by chlorophyll-*a*.

These observations suggest that much remains to be learned concerning the dependence of yield of photosynthesis upon wavelength, and that the yield near the long-wave end of the absorption spectrum may be of especial interest in connection with the manner of action of the different pigments. For both *Chlorella* and *Porphyridium* [and also for the diatom *Navicula minima;* cf. Tanada (39)], there is a steep decline in yield, beginning before the long-wave termination of light absorption, and well within the red absorption band of chlorophyll-*a*. The position of the decline is modifiable by temperature and by supplementary light, and probably also by other factors. Although the first evidence for green and for red algae suggests that the location of the decline is typically quite different for these two types of algae, adjustment of conditions can bring the decline to nearly coincide for these two types of algae, and this encourages us to seek a common interpretation.

No fully satisfactory explanation has yet been offered for the decline in yield within the red absorption band of chlorophyll first observed for *Chlorella*. Some of the proposed interpretations have been discussed by Rabin-

owitch [(36), Vol. II, part 1, pp. 1154–58). One possibility that has sometimes been considered is that the long-wave limit of maximum yield may represent the minimum energy per photon sufficing for the primary photochemical step in photosynthesis, and that a small deficit of excitation energy might be made up by thermal vibration, and hence that higher temperatures might extend the range of maximum yield to slightly longer wavelengths. It is noteworthy that the observed effect of temperature is opposite to this prediction. In any case, the speculation lacked physical basis, since absorption at any frequency within the red band system of chlorophyll should lead to the first excited state, regardless of differences in vibrational levels at the ground state, and any vibrational contribution to the excited state would be of such short duration that it could add nothing to the energy available for photochemical action.

It may be significant that the fluorescence of chlorophyll also shows a decline in yield within the red absorption band [Forster & Livingston (24, p. 1319)], and such a decline in yield is also known for other dyes. One is tempted to seek a common explanation for the long-wave decline in yield of both photosynthesis and fluorescence. A study of the fluorescence of dye solutions led Lavorel (31) to suggest such an interpretation. He found that the decline in yield of fluorescence on the long-wave end of the absorption spectrum was a function of concentration. In dilute solutions the decline was negligibly small, while at high concentrations it became very marked. The shape of the absorption spectrum was also a function of dye concentration. From an assumption that dimerization takes place at higher concentrations, and that the nonfluorescent dimer has two absorption bands, one on each side of the band of the monomer, Lavorel derived a quantitative explanation for these observations. Most of his observations were made on solutions of fluorescein. He also obtained similar results with thionine and with chlorophyll-*a*. He therefore drew a comparison between the behavior of photosynthesis and fluorescence on the long-wave side of the red absorption band, and suggested that dimerization might account for the decline in yield in both cases. The dimer would compete with the monomer for absorption, and would also act as an "energy trap" for energy absorbed by the monomer. Lavorel suggests reasons for anticipating that the effect would be greater at higher temperatures.

Since the dimer also shows a band on the short-wave side of the monomer band, there might be competition between dimer and monomer for absorption on the short-wave side of the monomer band, as well as on the long-wave side. This might explain the reduced yield for *Chlorella* photosynthesis, beginning [according to Emerson & Lewis (22, Fig. 5)] at about 580 mμ, a region where there is no apparent reason for a decline in yield, since it seems unlikely that absorption by carotenoids could extend so far toward long wavelengths.

Lavorel's suggestion that a variable fraction of the chlorophyll in living cells may be present in a dimeric and inactive form seems to offer at least a

qualitative explanation for the decline in yield of both photosynthesis and fluorescence in the longer wavelengths of the zone of light absorption. It would also be applicable to the corresponding long-wave decline in yield of the quinone Hill reaction, reported by Ehrmantraut & Rabinowitch (14, p. 76) for whole *Chlorella* cells. It promises to account for the effect of temperature, as far as photosynthesis is concerned. If higher temperatures increase the fraction of chlorophyll in dimeric form, then the effect of temperature on yield of fluorescence at longer wavelengths should be the same as on that of photosynthesis. It is not yet known whether this is so.

Lavorel does not speculate on the meaning of the effects of supplementary light on photosynthesis described by Emerson and co-workers. It seems unlikely that supplementary light could have similar effects on the yield of fluorescence.

In order to account for the effects of supplementary light, Emerson (15, 19) has suggested an entirely different explanation for the long-wave decline in yield of photosynthesis. Since the long-wave limit of maximum yield of *Chlorella* photosynthesis seems to coincide with the long-wave limit of absorption by chlorophyll-*b*, he suggests that maximum yield of photosynthesis requires not only excitation of chlorophyll-*a*, but also excitation of some other pigment having its first excited state at an energy level higher than that of the first excited state of chlorophyll-*a*. The low yield for light beyond the range of absorption by chlorophyll-*b* can be raised to normal by supplementary illumination of shorter wavelengths and, as was mentioned above, of the shorter wavelengths so far tested, those which were most effective were in spectral regions where absorption by chlorophyll-*b* is strong compared to that by chlorphyll-*a*.

The red, brown, and blue-green algae lack chlorophyll-*b*, but contain other pigments which might meet the proposed requirement. The long-wave limit of maximum yield for these algae, and their responses to supplementary light, should serve as tests for Emerson's hypothesis.

The chlorophyll-*c* which is present in brown algae and diatoms is the nearest analog to the chlorophyll-*b* of higher plants and green algae. Its red absorption maximum is probably at about 640 mμ, slightly toward shorter wavelengths than the maximum for chlorophyll-*b*. It is of course speculative to conclude from this that the long-wave limit of absorption for chlorophyll-*c* is also shorter than the long-wave limit for the *b* component, but this is certainly possible. Tanada's measurements of the quantum yield of photosynthesis for the diatom *Navicula minima* (39, Fig. 4) show the decline toward long wavelengths beginning at a point about 5 mμ shorter than the long-wave limit for *Chlorella* shown in the measurements of Emerson & Lewis. The difference is too small to be decisive, but it is in the predicted direction.

The red algae are not known to contain any chlorophyll component with a first excited state higher than that of chlorophyll-*a*. The chlorophyll-*d* discovered by Strain and Manning in certain red algae has its red absorption

maximum at about 720 mμ, so it would not meet the requirements. On the other hand, the phycoerythrin and phycocyanin of red algae might serve the purpose suggested for the chlorophyll-*b* of green algae, and Brody's measurements of the quantum yield for *Porphyridium* under various conditions provide data which offer several opportunities for testing the hypothesis. The general evidence that for red algae the range of maximum yield does not extend as far toward long wavelengths as it does in green and brown algae implies that the role of second pigment is being filled by a pigment or pigments having absorption bands that do not extend as far toward long wavelengths as those of chlorophylls-*b* or *c*. The long-wave limits of absorption by phycocyanins and phycoerythrins in living cells cannot be estimated with certainty, but the typical phycocyanins of blue-green algae probably absorb toward long wavelengths about as far as chlorophyll-*b* [cf. Fig. 1 of Emerson & Lewis (21)], the allophycocyanin of red algae perhaps not so far. Rabinowitch [(36), Vol. II, part 1, p. 666] shows figures from Svedberg and Eriksson that indicate appreciable absorption by solutions of phycoerythrins extends only to about 600 mμ. The tails of bands extending toward longer wavelengths probably represent scattering. It seems unlikely that in living cells absorption by phycoerythrin could extend as far as 644 mμ, the wavelength where Brody found maximum yields of photosynthesis for typical samples of *Porphyridium* cells. However, the phycocyanins (believed to be generally present in red algae) could serve as second pigment in this region. It would be plausible to suppose that differences in the long-wave absorption limits of the different phycocyanins could account for the considerable spread in the long-wave limit of maximum quantum yield for different red algae indicated by the work of Haxo & Blinks, Yocum & Blinks, and Brody. All these authors mention modification of the yield at long wavelengths by exposure of red algae to certain colors of light, and these effects would imply changes in the coupling between phycocyanins and chlorophyll-*a*.

The effects of shorter wavelengths of supplementary light during measurements of quantum yield at longer wavelengths have not yet been investigated with red algae, beyond the point of demonstrating, that shorter wavelengths do increase the yield from long wavelengths, just as they do in the case of *Chlorella*.

The evidence concerning blue-green algae seems to be conflicting. Emerson & Lewis (21, Fig. 4) show a curve for quantum yield of *Chroöcoccus* photosynthesis extending slightly further toward long wavelengths than the yield for *Chlorella*. Although the absorption maximum for phycocyanin falls at a wavelength considerably shorter than the red maximum for chlorophyll-*b*, the long-wave side of the phycocyanin band is not as steep, and absorption may very likely extend further toward long wavelengths than is the case for chlorophyll-*b*, so longer wavelengths should still suffice to produce the first excited state of phycocyanin. In contrast to this favorable evidence from *Chroöcoccus*, Haxo & Blinks mentioned that in some trial

experiments with *Oscillatoria* they found evidence of a decline in yield of photosynthesis at much shorter wavelengths.

A serious weakness of Emerson's suggestion to explain the long-wave limit of maximum yield of photosynthesis and the effects of supplementary light, is that it fails to include an interpretation of the corresponding long-wave decline in yield of fluorescence. It is also in conflict with the now widely accepted view that chlorophyll-*a* serves as an "energy trap" for the excitation energy of all pigments having first excited states at higher levels, and that chlorophyll-*a* alone transfers excitation energy directly to the biochemical steps in photosynthesis. The principal support for this viewpoint comes from the work of Duysens (12, 13) on sensitized fluorescence. While Duysens has stated his conclusions regarding the high efficiency of energy transfer from all pigments (including chlorophyll-*b*) to chlorophyll-*a*, with considerable certainty, it may be noted that Terenin & Ermolaev (40) characterized Duysens' evidence as largely indirect, and dependent upon estimates of re-absorption of primary and secondary fluorescence which are at best rather uncertain. It remains possible that the funneling of excitation energy into chlorophyll-*a* may not be as complete as is indicated by Duysens' estimates.

Additional data are needed, on the behavior of both photosynthesis and fluorescence near the long-wave limit of light absorption, before it can be said that the range of maximum yield must be limited by the same factors in both cases. Present evidence does not exclude the possibility that the extent of maximum yield toward long wavelengths is limited by different factors in the case of photosynthesis and fluorescence.

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PHYSIOLOGY OF SALT TOLERANCE[1]

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An understanding of the physiology of salt tolerance of plants is important for an effective approach to the salinity problem which is of increasingly widespread occurrence. The excessive accumulation of soluble salts in the rhizosphere is a potential if not an actual limiting factor for the productivity of irrigation agriculture everywhere. Hayward (52) has estimated that as much as 25 to 30 per cent of the irrigable land in the United States is subject to some degree of salt or sodium accumulation. Recently, widespread interest in the beneficial effects of supplemental irrigation during periods of drought has extended the concern with salt problems to the humid eastern sections of the country. Salt-spray damage and salt-water intrusions along the coast, and salinity problems in green house operations represent additional focal points of interest in salinity.

Plant-growth responses on saline soils have been discussed by Hayward & Wadleigh (60), Hayward (53), Grillot (46), and Hayward & Bernstein (54). The present account will, therefore, be restricted to an evaluation of physiological factors involved in salt tolerance rather than to a comprehensive review of salt tolerance literature.

The effects on plant growth of excessive concentrations of soluble salts in the root medium may be mediated by osmotic inhibition of water absorption, by specific effects of the constituent ion(s) in the saline media, or a combination of the two. Furthermore, specific ion effects may involve direct toxicity or a variety of nutritional effects. It will be more convenient to discuss osmotic and specific ion effects separately, although the two may frequently operate simultaneously.

OSMOTIC EFFECTS

Foremost in the several lines of evidence which may be adduced to support the concept of osmotic effects are the studies involving isosmotic additions of various salts to a base nutrient solution. To the extent that different salts produce equivalent growth depressions at equal osmotic pressures, the evidence is consistent with the osmotic inhibition theory. Any deviation in growth response for one salt compared to another indicates the presence of a specific ion effect in addition to the osmotic effect. Thus, Gauch & Wadleigh (42) concluded that the progressive decrease in growth of red kidney bean plants with increasing osmotic pressure in the range 1 to 4 atm. was directly and primarily related to osmotic pressure when NaCl,

[1] The survey of literature pertaining to this review was concluded in September, 1957.

Na_2SO_4, and $CaCl_2$ were the salts added to the base nutrient solution. With $MgCl_2$ and $MgSO_4$, however, additional specific effects of magnesium resulted in greater depressions of growth than occurred with the first three salts. This technique has been frequently employed to distinguish between osmotic and specific ion effects [see also Hayward & Long (55); Magistad *et al.* (74); Hayward & Spurr (59); Wadleigh & Gauch (100)].

Alternative explanations for the similarity of growth response on isosmotic solutions of different salts must assume equivalent effects on the growth processes of the plant of such ions as sodium and calcium and of chloride and sulfate. Such an assumption is extremely dubious in light of the well-known opposing effects of sodium and calcium on living cells and colloidal systems in general [Chambers & Reznikoff (23)]. Iljin's experiments (61) on changes in stomata and guard cells in epidermal strips of leaves, strikingly demonstrate the differential salt sensitivity of glycophytes and halophytes. The glycophytes were more sensitive to Na than Ca, and the reverse was true for halophytes. Calcium antagonized the effects of sodium.

The progressive decrease in growth with increasing osmotic pressure of the external solution can be explained in terms of classical osmotic theory by the decrease in diffusion pressure gradient between the medium and the plant. Eaton (37) has questioned the actuality of this change in diffusion pressure gradient on the basis that the osmotic pressure of the leaves or aboveground parts of plants increases as the salinity of the medium increases. This results in the maintenance of essentially a constant gradient between medium and plant. However, this argument does not take into consideration the osmotic properties of the root as an integral part of the water-absorbing and transmitting system. Limited observations on the osmotic pressure of roots indicate that these organs possess markedly lower osmotic pressures than the aboveground parts and do not vary nearly so much as the latter with increasing salinity of the root medium (Table I). It is recognized that the determination of osmotic pressure in expressed sap of frozen roots is subject to a number of errors: the mixing of cell sap with solution in "outer space" (39); the mixing of cell sap with the usually more dilute solution from xylem elements; adherence of rinse water to roots not completely removed by spin-drying; etc. Even allowing for appreciable influences by these factors, the general conclusions cited would remain valid and the diffusion gradient between medium and roots would decrease appreciably as the osmotic pressure of the external solution increases. The osmotic pressure differences between the leaf or root sap and the root medium are given for all paired leaf-root samples and for paired values omitting the three most sensitive crops (Table I). Similar results from both computations indicate the relative stability of leaf-medium gradients and the progressive decline in root-medium gradients with increasing salinity. In view of the possible errors inherent in the determination of

TABLE I

OSMOTIC PRESSURES OF EXPRESSED SAP OF LEAVES AND ROOTS OF PLANTS GROWN ON SAND CULTURES WITH GRADED ADDITIONS OF NaCl TO THE BASE NUTRIENT SOLUTION*

Plant	O.P. of NaCl for 50% growth decrement†	Part	Osmotic pressure of expressed sap in atm.				
			Added NaCl, atm.				
			0	1.25	2.50	3.75	5.00
Onions	1.25	Leaves	10.4	8.6	11.3	—	—
		Roots	3.6	4.1	4.7	—	—
Cucumbers	1.25	Leaves	6.9	6.8	8.1	9.4	—
		Roots	2.9	2.6	3.4	2.9	—
Peas	2	Leaves	10.5	14.2	16.8	—	—
		Roots	5.1	10.4	9.3	4.7	—
Peppers	3.75	Leaves	12.1	12.4	16.7	—	13.7
		Roots	5.4	5.5	5.4	5.8	7.0
Beans	4	Leaves	8.3	9.1	9.3	11.3	11.4
		Roots	3.9	4.0	3.6	3.0	3.2
Lettuce	4	Leaves	5.3	6.3	8.4	9.0	10.7
		Roots	3.0	3.4	3.9	4.4	6.6
Cabbage	4	Leaves	9.9	11.9	12.7	11.3	11.9
		Roots	3.3	3.9	5.1	4.2	4.6
Spinach	‡	Leaves	8.0	8.5	9.7	10.3	12.1
		Roots	2.8	3.2	2.9	3.6	4.0
Tomato	‡	Leaves	10.3	10.4	10.3	9.5	9.5
		Roots	2.9	6.9	—	4.1	5.0
Beets	‡	Leaves	10.3	13.4	15.2	16.4	18.3
		Roots	2.6	3.2	3.4	3.7	4.0
			Calculated for all paired leaf-root values				
Average O.P. of leaves			9.20	10.16	12.02	11.03	12.51
O.P. leaves—O.P. solution§			8.7	8.4	9.0	6.8	7.0
Average O.P. of roots			3.55	4.72	4.63	3.70	4.91
O.P. roots—O.P. solution§			3.0	3.0	1.6	−0.5	−0.6
			Calculated for all paired leaf-root values omitting onions, cucumbers, and peas				
Average O.P. of leaves			9.17	10.28	12.00	11.30	12.51
O.P. leaves—O.P. solution§			8.7	8.5	9.0	7.0	7.0
Average O.P. of roots			3.41	4.30	4.05	3.83	4.91
O.P. roots—O.P. solution§			2.9	2.6	1.0	−0.4	−0.6

* Species listed in approximate order of increasing salt tolerance in this test.

† Based on fresh weight of tops.

‡ At 5 atm. level, growth reduced 36, 5, 0% for spinach, tomatoes, and beets, respectively.

§ O.P. solution = O.P. of added NaCl + 0.5 atm., O.P. of base nutrient solution.

osmotic pressure of root sap, no special significance should be attached to the negative values for the root-medium gradients at the two highest salinity levels.

Mayr (78) determined the osmotic pressure of epithelial cells of wheat, barley, and oat roots, using mannitol to observe incipient plasmolysis directly under the phase contrast microscope. With few exceptions, the osmotic pressure at incipient plasmolysis was found to be about 3.5 atm. This value is in good agreement with data for expressed root sap of other crops cited in Table I. The concept of outer space [Epstein (39)], moreover, suggests that water absorption by cells from the external medium may occur throughout a major portion of the root and not be restricted to the epidermal cell layer. The view has even been advanced that outer space may constitute an interconnected system throughout the plant [Kramer (68)]. If this were the case, then Eaton's concept (37) that osmotic pressure of the leaves may be effective in water absorption would be justified inasmuch as the leaves may be essentially in contact with a more or less modified root medium via outer space. It is difficult, however, to reconcile this view with the well-known selectivity of plants in the absorption of nutrients and water from the root medium. Black (18) found that the salt content of *Atriplex hastata* was much less than the quantity originally present in the volume of water absorbed. To realize the impossible consequences of the assumption of mass absorption of the external medium by the plant, one need only consider that a plant with a water requirement of 500 growing on a medium containing 50 m.eq./l. of salt would accumulate 2500 m.eq. of salt per 100 gm. of dry plant matter, if the root solution were absorbed *en masse*. This level of salt accumulation is almost an order of magnitude greater than that usually found in crop plants, even when grown on saline media.

It, therefore, seems likely that there exists within the plant root a limiting layer or layers of cells which delimits the inner boundary of outer space. These cells would transmit water by osmotic processes deeper into the plant tissues while the absorption of certain ions is restricted, thereby building up in the outer layers a concentration which prevents further diffusion of this ion species into the plant, or even promotes an outward diffusion into the root medium to re-establish equilibrium. The osmotic properties of the cell layers bounding outer space would then become the limiting factor in transmitting the diffusion pressure deficit of the aboveground parts to the root medium.

Bernstein & Pearson (16) have shown that tomato and pepper plants can survive high osmotic pressures of up to 12 atm. for a day or two. Yet the same authors showed that growth of peppers was reduced to almost zero if osmotic pressure is maintained continuously at 6 atm. This corresponds to the osmotic pressure of the root of pepper plant rather than to that of the leaves (Table I). On the other hand, Tagawa (92) has found

that absorption of water by detopped bean roots ceases at an osmotic pressure of the external solution of 1.94 atm. whereas in the intact plant an osmotic pressure of 14.68 atm. is required to stop water absorption. The difference between the intact and detopped plants of 12.74 atm., Tagawa attributes to the suction exerted by the tops. Over a short period of time, high osmotic pressures in the root medium may perhaps reversibly alter the root and cause it to function purely as a wick in absorbing and transmitting water to the xylem elements. Sustained performance of this type, however, cannot apparently be maintained.

The qualitative aspects of plant growth on saline media are not without significance in relation to the question of osmotic inhibition of growth. Many workers in this field [Eaton (37); Hayward & Wadleigh (60)] have noted the progressive stunting of plants and the attendant smaller, darker green leaves which in most cases resemble drought symptoms very closely. They support the view that water deficit induced by high osmotic pressure of the root medium is the factor restricting growth.

More directly, Hayward & Spurr (58) have shown the nearly equivalent effects of isosmotic solutions of salts, sucrose, or mannitol on the absorption of water by corn roots. Eaton (36), using a divided root system technique, demonstrated greater water absorption from dilute than from concentrated solutions. Isosmotic solutions of chloride, sulfate, or additional nutrient salts were equally inhibitory to water absorption, indicating that osmotic effects were primarily involved. Long (72) observed similar effects using approach-grafted tomato plants.

The data in Table I are also instructive from another point of view. Plant species are arranged in approximate order of increasing salt tolerance as evidenced in this particular greenhouse trial. There is no apparent correlation between salt tolerance and osmotic pressure of expressed sap of leaves or roots. The extremely salt-sensitive peas have the highest osmotic pressures for example, and even show the most striking increase in osmotic pressure with increasing salinity. The increased osmotic pressure probably is related to the degree of growth inhibition and the greater accumulation of solutes in the cells of the stunted plants. The osmotic pressures of plant parts, however, represent only a measure of the potential intensity factor for the absorption of water. The volume of water absorbed will depend not only on the diffusion pressure gradient, permeability, and other factors relating to the intensity of absorption, but also on the water-absorbing root area. Repp (87) has discussed the importance of root characteristics, shoot-root ratios, and related factors in relation to salt tolerance. No quantitative estimates of the relative importance of these factors have, however, been made either in relation to general salt tolerance theory or even for a given plant species. It may be expected that shallow-rooted plants or those with large top-root ratios and, therefore, having a low capacity for water absorption in relation to transpiration will exhibit poorer salt tol-

erance than deeper-rooted species or those with low top-root ratios, other factors being equal. A decrease in top-root ratio with increasing salinity has often been observed [Eaton (37); Bernstein & Pearson (16)]. This appears to be a significant feature in the adjustment of a plant to salinity inasmuch as the proportion of water-absorbing to transpiring organs is thereby increased.

Efficiency of water use under conditions of limited moisture availability may also be a factor in salt tolerance. Eaton (35) found that the water requirements of wheat and Australian saltbush decreased with increasing salinity, i.e., less water was used per gram of dry matter produced. In a later study, Eaton (37) found a decrease in water requirement for mixed cultures of eight crops at moderate salinity levels but an increase at higher levels of salinity. The significance of varied water requirements of crops in relation to salt tolerance cannot be evaluated at this time.

It has been noted that plants developing under cool weather conditions may appear to be only slightly affected by salinity but a sudden onset of hot weather will often produce marked injury symptoms such as wilting and leaf scorch [Bernstein & Ayers (9)]. This phenomenon can be explained on the basis of relatively slight modification of the top-root ratio and leaf characteristics under low moisture-stress conditions associated with low evaporation. This results in a plant which is poorly adapted to function under high evaporative conditions when water availability is restricted by salinity.

Studies on the interaction of soil salinity and moisture tension provide another argument in support of the osmotic inhibition theory. Wadleigh and co-workers (97, 103) demonstrated that the growth of beans and guayule was a function of total soil-moisture stress regardless of the relative contributions of osmotic pressure or moisture tension. Total soil-moisture stress was defined as the sum of osmotic pressure and moisture tension. Osmotic pressure was the predominant factor in total soil-moisture stress in the saline, low-tension treatments while moisture tension predominated in the nonsaline, high-tension treatment. Figure 1 represents characteristic soil-moisture stress curves for nonsaline soil on wet and dry regimes compared to the stress curve for a saline soil. When the areas under two such dissimilar stress curves as B and C were equal, growth of the plants was found to be reduced to very nearly equal degrees. This indicates that moisture availability is apparently the limiting factor for plant growth on saline soil.

The nature of curve C (Fig. 1) accounts for the general lack of wilting symptoms in plants on saline soil, despite the fact that their growth may be limited by moisture availability. Because of relatively high osmotic pressures in the soil solution, moderate moisture stress is maintained throughout the irrigation cycle, with no abrupt changes in stress, provided proper irrigation frequencies are employed. Decreased rates of water absorption

characteristic of plants on saline soil [Eaton (35, 36, 37)] contribute to the relative uniformity of moisture stress values. Because of the lack of wilting symptoms in plants on saline soil, particular care is required that irrigations are not overly delayed. Less frequent irrigation aggravates moisture stress and intensifies the growth inhibition associated with a given level of salinity [Wadleigh *et al.* (97, 103)].

Because of the high osmotic-pressure component in moisture stress of saline soils, the available moisture supply in a given soil generally decreases as salinity increases. Fedorovskii (40) found this relationship obtained for

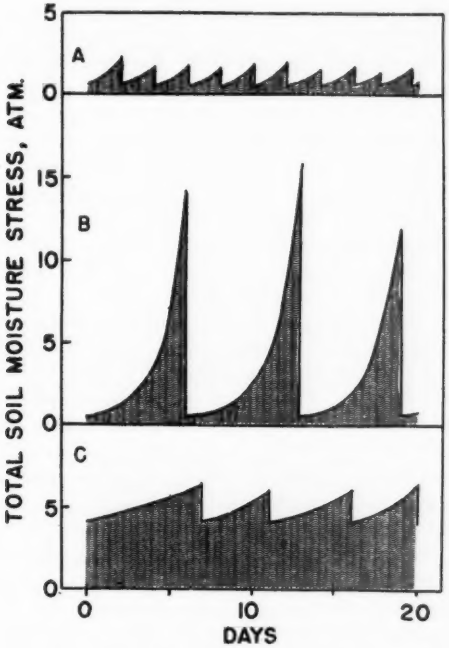

FIG. 1. Total soil moisture stress in a frequently (A) and infrequently irrigated (B) nonsaline soil and in a saline soil (C).

a number of crop plants tested, but halophytes (*Salicornia, Salsola*) were able to bring the moisture content of soils to as low, or even a lower level, in the presence of salinity, as in its absence. Wadleigh, Gauch & Strong (104) determined root penetration into soil layers of increasing salinity. The roots removed less water as the salinity increased, resulting in the

development of an essentially uniform osmotic pressure throughout the root zone for each crop as follows: bean, 7 to 8; corn, 10.5 to 11.5; alfalfa, 12 to 13; and cotton, 16 to 17 atm.

For most plants, therefore, saline soils should be irrigated when the soil moisture content is appreciably above the permanent-wilting percentage of the soil as determined in the nonsaline condition. The higher the salinity, the greater is the residual soil moisture which is unavailable to the plant.

Recently, Gingrich & Russell (45) and Danielson & Russell (32) studied the effects of variations in soil-moisture tension and osmotic pressure on root elongation and ^{86}Rb absorption by corn seedlings. The greater effect of moisture tension compared to equivalent osmotic pressures on both processes was attributed to a limiting rate of moisture transmission and ^{86}Rb diffusion as moisture film thickness decreased in media adjusted to higher moisture tensions. Bernstein & Pearson (16) determined the effects of controlled variations in osmotic pressure on the growth of pepper and tomato plants. Average osmotic pressures of 4 atm. were maintained using four different time-stress curves one of which ranged up to 12 atm. With peppers, no significant effect of osmotic pressure range was observed while with tomatoes growth was significantly poorer when high osmotic pressures occurred. In integrating variable osmotic-pressure and moisture-tension values over a period of time, it is, therefore, obvious that marked departures from a linear growth response must be taken into account. Wadleigh & Gauch (101) used the rate of leaf elongation of cotton as a measure of growth in relation to total moisture stress. This relationship during an irrigation cycle was expressed empirically as a second degree function. The derivative of this function, at which growth was zero, approximated 15 atm. and was consistent for different irrigation cycles during the growth of a given leaf and for different leaves on the same plant or different plants.

In addition to the plant characteristics, such as top-root ratios, environmental factors may also influence the development of moisture deficits in plants. Magistad *et al.* (74) found the growth of onions on saline media to be much more severely depressed in hot, dry climates than in cooler, more humid areas, whereas the salt tolerance of beets was affected to a much lesser degree. Seasonal effects may also be of similar origin. Van Dam (31) has reported autumn cauliflower to be more salt tolerant than spring-grown cauliflower. This evidence supports the concept that moisture deficits in plants on saline soil largely control growth rates and yields. Nevertheless, it is significant that investigations under the very dissimilar climatic conditions of California, North Africa, and Holland, have generally yielded very comparable data on the relative salt tolerance of plants [Hayward & Bernstein (54)].

Ratner (83) studied the influence of transpiration on the absorption of minerals by plants grown on saline soils. He distinguished between transpiration effects on low-salt and high-salt plants. In the former, transpiration

rate was relatively ineffective and Ratner concluded that the salts may have been actively absorbed by root cells and translocated independently of the transpiration stream. In high-salt plants, however, transpiration was effective in increasing salt uptake and Ratner postulated that salts bypassed the root cells and were translocated in the xylem. Ratner concluded that high transpiration rates of plants on saline soil was damaging because of increased salt absorption. It seems more likely, however, that high transpiration simply increases the moisture deficit in the plant, thereby impairing growth. Increased salt absorption may occur, but still may not be responsible for decreased growth.

In addition to the direct effects of decreased water absorption on plant growth, moisture deficits in the plant may affect growth indirectly through changes in the rates of metabolic processes. Few direct determinations of the effects of salinity on plant metabolism have been made although such effects have sometimes been postulated. Kling (66) tested the effects of increased concentrations of a balanced salt solution (Van't Hoff-Richter) on the growth and photosynthetic rates of alfalfa and tomato. He noted the usual progressive inhibition in growth with increasing salinity, the increase in succulence and the darker green color of the leaves. Photosynthetic rates, however, measured in the Warburg apparatus and expressed on a leaf area basis, were regularly higher for the plants on the saline treatment than for the control plants. The differences in rates were greatest (about twofold) with light intensities of 5000 to 6000 lux and were smaller at lower and higher light intensities. In discussing the effects of salinity on photosynthesis and accumulation of carbohydrates, Kling notes several instances of marked carbohydrate accumulation, especially in cotton, but concludes the data are not generally consistent and vary greatly with conditions of the experiments.

Although the relationship of metabolic rates to soil moisture status may not be comparable to the effects of salinity, the studies of Simonis (89, 90) appear worthy of mention. Simonis emphasizes the importance of the adaptation of plants to limited soil moisture conditions. Thus, a plant subjected throughout its growth to a limited moisture supply may be affected differently from one which develops with abundant available moisture before the moisture supply is reduced. With plants grown on soil cultures at 80 per cent and 30 per cent of water-holding capacity, photosynthesis was regularly higher for plants of the dry treatment than the wet when the rates were expressed on leaf area, fresh weight or dry weight bases. Similar findings were obtained for four species. Only under extreme moisture deficiency did plants of the dry treatment have a lower photosynthetic rate. Growth was markedly depressed under the dry treatment compared to the wet and Simonis (89) relates this to a disproportionately greater growth of roots than of tops during the early growth stages. The similarities between the effects of maintained moisture deficiency and those of salinity

previously described are evident. Respiration rates were variably affected by limited moisture supply, some species having higher and others having lower rates on the dry treatment than on the wet.

Stocker (91) studied transpiration and assimilation rates of xerophytes and halophytes in the field. While xerophytes generally exhibited a marked decrease in net assimilation early in the day when soil-moisture supply was low, halophytes adequately supplied with water but on highly saline soils maintained a high net assimilation rate throughout the day.

From the limited evidence available, reduced growth associated with decreased moisture availability or salinity is not necessarily associated with a decrease in photosynthetic rate as measured in the Warburg [Kling (66)] or Van der Paauw apparatus [Simonis (89, 90)].[2] If growth rate decreases while net assimilation is maintained, an increased accumulation of reserve carbohydrates might be expected. This has been observed to a very marked degree in the case of carrots [Bernstein & Ayers (10)] but to only a very slight extent in onions [Bernstein & Ayers (11)]. Wadleigh and coworkers (97, 102) report a decrease in starch in bean plants with either high moisture tension or osmotic pressure. Sugars and hemireserves were variably affected. Gauch & Eaton (41) found that sucrose and starch increased markedly in barley plants on saline media, and Hayward & Long (55) reported salinity increased starch accumulation in basal stem tissues of tomato plants.

It may be said in summary, that while the osmotic inhibition theory is supported by cogent evidence, the specific features and details remain obscure. Recent developments in the relationship of plant roots to the external medium involved in the concept of outer space have raised additional questions, but also afford a new approach to the problem.

SPECIFIC ION EFFECTS

Nutritional Effects

Of the large number of ionic species which may occur in solution or in the adsorbed state in soils, relatively few contribute to salinity in a given saline soil. The cations, Ca^{++}, Na^{+}, and Mg^{++} and the anions Cl^{-}, $SO_4^{=}$, HCO_3^{-}, and $CO_3^{=}$ generally predominate, although in any given soil the proportions may vary considerably. The occurrence in restricted areas of high concentrations of NO_3^{-} or K^{+} is not unknown and in greenhouse operations, or in intensively farmed areas, excessive fertilization may further increase the variety of ionic species present in excess. It is not surprising that a tenfold increase or more of a given ion over its concentration in a

[2] It should be noted that metabolic rates of organs in closed vessels may only be a measure of potential photosynthetic or respiratory rates. Actual rates in intact plants may be quite different because of changes in environmental and internal factors (e.g., wilting and stomatal closure).

nonsaline soil may produce occasional nutritional unbalance. In fact, one is rather impressed by the relatively infrequent occurrence of such effects. With regard to saline soils, one is concerned with the occurrence in excess of a salt or salts in an otherwise normally fertile or fertilized soil. In many of the earlier salinity studies, especially those concerned with germination, single salt solutions were used, provoking many specific salt effects which disappear when a more balanced salt solution is employed. Thus, more recently, Ota, Ogo & Omori (79) found that germination of wheat was poorer on NaCl solutions than on isosmotic glucose solutions but that isosmotic dilutions of sea water were less toxic than NaCl. Uhvits (95), likewise, found NaCl to be more toxic to germinating alfalfa seed than isosmotic solutions of mannitol.

Cations.—Considering the cations first, saline soil solutions usually contain Ca^{++}, Na^{+}, and Mg^{++} in which the proportion of Na^{+} cannot be excessive if deterioration of soil structure characteristic of sodium or alkali soils is to be avoided (96). While excessive concentrations of Mg^{++} may be toxic because of excessive absorption of Mg accompanied by greatly decreased absorption of Ca and K, such an effect is usually eliminated by the simultaneous presence of moderately high concentrations of Ca^{++} along with Mg^{++} [Hayward & Wadleigh (60)]. High concentrations of Ca^{++} may also produce nutritional unbalance in some species unless compensated by some other ion such as Na^{+} or K^{+}. Bernstein & Ayers (10), in studying the salt tolerance of five varieties of carrots, observed relatively poor yields at a given salinity level for those varieties which tended to accumulate more Ca and less K. Salinity, achieved by addition of $CaCl_2$ and NaCl to the irrigation water, increased the absorption of Ca and depressed the absorption of K. Those varieties which on nonsaline soil accumulated less K were less resistant to the nutritional unbalance induced by these salt treatments. It is significant that of a large number of crops tested by this artificially salinized field plot technique, only one or two evidenced any nutritional disorders of this type. Bean plants accumulate such high levels of Ca that a nutritional component as well as the osmotic one may have operated to decrease yields [Bernstein & Ayers (9)]. Van den Berg (7) has suggested that salt tolerance is inversely related to the level of calcium accumulation by plants. The salt in the inundated areas of Holland was principally NaCl from sea water and the effects may have been partly related to sodium tolerance as well as to general salt tolerance.

In greenhouse operations, heavy applications of fertilizers may produce other specific ion effects not commonly encountered in the field. Thus, Kofranek, Lunt & Hart (67) observed specific effects on the growth of chrysanthemums associated with high levels of ammonium or magnesium salts. Cation effects in water- or sand-cultures have generally been more striking than in soil cultures (8) undoubtedly because of the buffering action exerted by the exchangeable cations in soils, primarily Ca^{++}, in modifying

extreme cationic unbalance caused by high concentrations of Mg^{++}, K^{+}, or Na^{+}.

Anions.—While relatively few instances of cationic unbalance have been observed in saline soils, the situation is even more striking with respect to anions. Under nonsaline conditions, chloride may be present in only traces up to a few m.eq./l. and absorption by the plant will be relatively low. Under saline conditions, however, the chloride in the soil solution may reach values of 100 m.eq./l. or more and accumulation in leaves of plants may increase up to 150 m.eq./100 gm. or more [Bernstein *et al.* (9, 10, 12)]. Yet this large increase in external concentration of chloride and in the amounts absorbed exerts no marked effect on the absorption of the essential anions, phosphate, nitrate, and sulfate. Reifenberg & Rosovsky (86) used serial dilutions of a saline well water with various concentrations of added nutrients in a study of nutrient uptake by barley seedlings. They found little or no effect of chloride in concentrations up to 3000 p.p.m. on the absorption of nitrate or phosphate. They did observe, however, that an increase in phosphate or nitrate depressed chloride absorption. Arnold (1) found no specific effect of replacing part of the nutrient anions by chloride in the solution culture of tomato. The stability in nutrient anion absorption may be related to the specific absorption sites for these anions [Epstein (39)] but rapid conversion of anions into organic compounds may also be a factor in maintaining a high rate of absorption. This latter factor may also account for the apparently greater stability of nutrient anion absorption in the presence of other anions than appears to be the case for cations.

Gauch & Wadleigh (43), however, did find a depression in total N-concentration in bean plants when high concentrations of NaCl or $CaCl_2$ were added to the culture solutions. A small repression in N-uptake may, however, not be reflected in yields if the growth rate is reduced by high osmotic pressures. It is noteworthy that sulfate which is absorbed in much smaller quantities than chloride produces equivalent effects on growth of bean plants (42). Furthermore, other studies on tomato, barley, and bean plants indicate that salinity increased the total N-content of the plants [Hayward & Long (56); Gauch & Eaton (41); Wadleigh & Ayers (97)].

Although the uptake of chloride from added NaCl or $CaCl_2$ appears to be equal for most plants studied (43), stone-fruit trees take up about twice as much Cl per m.eq. of Cl in the nutrient solution from the Ca-salt than from the Na [Brown *et al.* (22)]. This appears to be a significant factor in the development of chloride toxicity in these susceptible species.

The sulfate ion generally restricts the absorption of Ca while promoting the uptake of Na [Hayward & Wadleigh (60)]. Calcium rarely reaches the point of deficiency although this may have been involved in sulfate-toxicity effects on some lettuce varieties [Doneen & Grogan (34)]. Zusman (108), however, found no evidence of Ca deficiency in citrus seedlings suffering from sulfate toxicity. The sulfate toxicity symptoms consisted of a yellow-

ing of the leaf margins which spread interveinally toward the midrib followed by necrosis. The S content of the leaves, expressed as SO_3, increased to about 1 per cent. By promoting the uptake of Na, sulfate may induce sodium toxicity in susceptible species [Brown *et al.* (22)].

Toxic Effects

Toxicity, as used in the present account, will refer to any inhibition in growth or function which appears to be related solely to the excessive accumulation of a specific ion. It is sometimes difficult to distinguish between such toxic effects and other specific ion effects. Indeed, some authors have considered all specific ion effects as toxicities. Excessive absorption of Mg, associated with lower absorption of Ca and K, may inhibit growth because of Mg toxicity, Ca or K deficiency, cationic unbalance or a combination of these factors. It seems best, therefore, to consider the effect of Mg and similar effects of excess Ca and K, as nutritional disturbances rather than toxicities.

Chloride.—Excessive accumulation of Cl causes characteristic leaf-injury symptoms and dieback in a number of fruit-crop plants. Hayward and co-workers (22, 57) have described such chloride injury in stone-fruit trees and almonds and demonstrated the specificity of this effect by comparison with isosmotic solutions of sulfate salts. Ayers (2) has shown specific leaf injury associated with chloride accumulation in avocado. Leaf-burn symptoms have also been described for citrus, grapes, pecans, and native tree species of Oklahoma and for a variety of subtropical fruit-crop plants and ornamentals including papaya, mangoes, sapote, cherimoya, and others [see Table II and Cooper *et al.* (26, 27)]. In this last group of plants (citrus, pecan, etc) no definitive experiments [except for lemon—Eaton (37)] comparing chloride solutions with isosmotic solutions of other anions have been performed, so that the causal relationship of chloride accumulation to injury is still only presumptive. The level of Cl accumulation at which symptoms develop is not too well defined. Uninjured leaves may sometimes have higher chloride accumulation than injured leaves of the same species [Brown *et al.* (22); Cooper *et al.* (28); Harper (51)]. Nevertheless, it is noteworthy that for most of these species, chloride accumulations in the leaves expressed on the dry weight basis at which toxic effects occur are rather similar (Table II). These values may be contrasted with chloride accumulation data for species not specifically sensitive to chloride. Such species may accumulate 4 per cent chloride or more without evidencing any specific burn symptoms [Bernstein *et al.* (10, 12)]. However, species which are quite salt-sensitive may develop leaf-burn symptoms which are not specifically related to chloride accumulation; i.e., these species may burn just as badly with isosmotic concentrations of sulfate salts [Bernstein (8)]. Extreme moisture deficits apparently may produce leaf-injury symptoms which are difficult to distinguish from chloride burns. Controlled

experiments will obviously be necessary to establish the relationship between chloride accumulation and leaf burn in species for which this has not as yet been done.

The mechanism of chloride toxicity remains unknown. The similarity of chloride accumulation levels at which injury occurs in chloride-sensitive species suggests that there is a common mechanism and that these species differ qualitatively from the nonsensitive species.

Comparing the use of chloride and sulfate fertilizers, various authors have explained chloride effects on the potato plant by decreased translocation of carbohydrates [Latzko (69, 70)] and lowered rates of photosynthe-

TABLE II

MINIMAL LEVELS OF Cl ACCUMULATION IN LEAVES ASSOCIATED WITH LEAF-BURN SYMPTOMS

Species	Cl accumulation in leaves % dry weight	References
Stone-fruit trees	0.6 to 1.0	Hayward *et al.* (57); Brown *et al.* (22)
Almonds	1.2 to 1.8	Brown *et al.* (22)
Pecans	1.0	Harper (51)
Citrus	1.0 to 1.5*	Cooper *et al.* (28, 29)
Avocados	0.5 to 0.9	Ayers (2); Haas (47); Ayers *et al.* (4); Cooper (25); Cooper & Gorton (27)
Grapes	0.5 to 1.2	Thomas (93); Ravikovitch & Bidner(84); Woodham (107)

* Lower levels of Cl may cause bronzing (Reference cited).

sis [Baslavskaja, see discussion in Hayward & Wadleigh (60)]. Chloride, however, does not appear to be specifically toxic to the potato [Bernstein *et al.* (12)].

Chloride accumulation in a given scion may be greatly modified by rootstock. Rootstock studies with citrus [Cooper *et al.* (28, 29)], avocado [Cooper (25); Haas (47)], and stone-fruit trees [Bernstein *et al.* (13)] suggest practical means for alleviating chloride accumulation and toxicity in these sensitive species. By comparing growth and chloride accumulation of stone-fruit trees and almonds on Lovell roots with alternate rootstocks, Bernstein *et al.* (13) determined that approximately 50 per cent of the decrease in growth of these trees on Lovell peach roots was caused by chloride injury while the remainder was due to other effects, presumably the osmotic pressure of the moderately saline soil solution.

Bicarbonate.—The bicarbonate ion in excess is generally toxic to plants although the sensitivity varies with the species. Wadleigh and co-workers have shown bean (98) to be more sensitive than beets (21) and Dallis grass

more sensitive than Rhodes grass (44). A characteristic Fe-chlorosis is one feature of bicarbonate excess. Porter & Thorne (80) studied the interaction of bicarbonate ion concentration and CO_2 tension and found the former more effective in causing chlorosis of beans and tomatoes. The chlorosis problem has been recently reviewed by Brown (20). The bicarbonate ion does not frequently occur in sufficient concentrations in irrigation waters to produce toxic effects although bicarbonate-induced chlorosis of apple orchards has been observed in Washington [Harley & Lindner (50)]. It has also been suggested that the bicarbonate ion, produced by the reaction of $CaCO_3$ with CO_2 and H_2O in calcareous soils, may be primarily responsible for lime-induced chlorosis [Wadleigh & Brown (98)]. Of more general concern in saline soils is the tendency for bicarbonate ion to cause precipitation of calcium in the soil, thereby favoring an increase in exchangeable sodium [Wilcox *et al.* (106)].

Boron.—Boron is toxic to plants in concentrations only slightly in excess of those required for optimum growth of a species. Wilcox (105) discusses the extent of the boron-toxicity problem and presents boron-tolerance data for crops based on the work of Eaton *et al.* (38). In saline soils containing excess boron, it is generally more difficult to reduce boron to a safe level than the other saline constituents [Reeve *et al.* (85)]. Like chloride, boron accumulation in plants is subject to considerable control by rootstock selection. This has been demonstrated for citrus by Cooper *et al.* (30) and for stone-fruits by Hansen (48, 49). Boron accumulates in highest concentration in the leaves, making foliar analysis particularly effective, especially since toxic levels of accumulation have been established for many species by Eaton *et al.* (38). The mechanism of boron toxicity in plants is not known.

Sodium.—The response of plants to excess sodium may be complicated by a number of factors. Indirect effects on plants produced by structural deterioration of sodic soils, direct toxic effects in the case of sodium-sensitive species, and nutritional effects may all be involved. Plants tolerant to sodium may be inhibited in their growth primarily by the poor physical condition of sodic soils which restricts moisture transmission and aeration and may physically impede root elongation and seedling emergence. Such sodium-tolerant crops which may be primarily affected by poor soil structure, include beets and Rhodes grass (17, 19), cotton (24), tomatoes (94), and some of the grain crops (81). Moderately tolerant crops, such as alfalfa (17, 24), clover (17), Dallis grass (19), and the majority of other crops, may exhibit a nutritional component in the over-all growth reduction as well. Most authors are agreed with Ratner (81, 82) that increasing levels of exchangeable sodium in the soil result in decreased absorption of calcium, and attribute the nutritional component of growth inhibition primarily to this factor. However, Bernstein & Pearson (17) have found appreciable reduction in growth of beans with essentially no decrease in calcium con-

tent. Schreiber, Davis & Overstreet (88) reported that twice the concentration of exchangeable hydrogen or magnesium, compared to exchangeable sodium, was required to effect calcium loss from radish seedlings.

Plants which are still more sensitive to sodium and exhibit characteristic leaf-burn symptoms when sodium accumulation in the leaves becomes excessive may be injured at still lower concentrations of exchangeable sodium, even before the unfavorable physical condition of the soil becomes evident. Avocado [Martin & Bingham (75)], citrus and stone fruits [Jones *et al.* (63); Martin *et al.* (76, 77)], and possibly other tree crops would be in this category. Sodium injury to avocados grown on culture solutions was demonstrated by Ayers (2), and Brown *et al.* (22) observed sodium burn of plum and Texas almond trees on sand cultures. Minimal sodium accumulation in leaves showing sodium-burn symptoms were: 0.5 per cent in avocado [Ayers (2)]; 0.3 to 0.4 per cent in almonds and plum [Lilleland *et al.* (71); Brown *et al.* (22)] In these cases, unlike those in which calcium deficiency may be involved, there is no appreciable change in the content of other cations or nutrients suggesting that the sodium itself is directly toxic to these species.

SALINITY AND THE STAGE OF PLANT DEVELOPMENT

The effects of salinity on a plant may vary depending on the stage of its development. Sensitivity may be quite different during germination than at later stages, and fruiting in some crops may be more or less affected than vegetative growth. Kling (66) emphasizes the importance of the initiation of photosynthetic activity in seedlings as a factor in increasing their osmotic pressure and salt tolerance. The premise that salt tolerance in established plants is generally greater than in germinating seed is, however, open to question.

Germination.—It is difficult to compare tolerance during germination with later stages because of the dissimilarity of criteria employed in the evaluation of growth. The first effective increments of salinity for a given crop generally retard germination with little or no effect on the ultimate number of seedlings which emerge. Higher levels of salinity aggravate the delay in emergence and also decrease final germination percentages [Ayers & Hayward (6)]. The usual criterion for germination is the percentage of emergence rather than the time required, although the latter may be more comparable to growth rates at later stages. The delay in emergence is evident from a comparison of the salinity levels required to effect a 50 per cent reduction in emergence at 7 and 15 days (Table III). Because of the emergence of additional seedlings on the saline soils during the longer germination period, higher levels of salinity are required to produce a 50 per cent decrement. Except for alfalfa, figures comparable to those in columns A and B, are obtained for estimates of salinity required to effect a doubling of the time required for emergence. In the case of alfalfa, salin-

ity appears to be twice as effective in delaying germination as in decreasing the ultimate percentage of emergence. The ratios of salinities required for 50 per cent decreases in germination and in crop yields, are given in the last two columns of Table III. Only sugar beet shows a value significantly below unity, indicating appreciably greater sensitivity during germination than during later growth stages. Corn, on the other hand, appears to be appreciably more tolerant during germination than at later stages.

The spottiness of stands frequently observed on saline soils has apparently influenced the belief that plants are generally more sensitive during

TABLE III

COMPARISON OF SALT TOLERANCE DURING GERMINATION WITH TOLERANCE DURING LATER GROWTH STAGES

Plant	$EC_e \times 10^3$‡ for 50% decrement* in seedling emergence		$EC_e \times 10^3$‡ for 50% decrement† in crop yield (C)	A/C	B/C
	7 days (A)	15 days (B)			
Bean, Red Kidney	3.7	8.0	4	0.9	2.0
Sugar beets	5.3	6.0	16	0.3	0.4
Alfalfa	8.7	9.0	8	1.1	1.1
Corn, field	9.4	14.0	6	1.6	2.3
Barley, California Marriout	13.6	19.6	16	0.8	1.2

* Based on data from Ayers & Hayward (6).

† Based on salt-tolerance list (96).

‡ $EC_e \times 10^3$ = electrical conductivity of saturation extract of soil in millimhos per cm. at 25°C. (96).

germination than at later stages [Ayers & Hayward (6); Kling (66)], a generalization which is not supported by the data in Table III. The surface inch of soil is frequently more saline than the soil below it because of evaporation and capillary rise of saline waters to the surface. This concentrating of salt is greatly aggravated in furrow-irrigated ridges or raised beds [McGeorge & Wharton (73); Wadleigh & Fireman (99); Bernstein & Fireman (14)]. The seed, therefore, is generally in a more saline environment than the established plant whose roots can utilize less saline portions of the soil profile [Wadleigh & Fireman (99)]. Furthermore, evaporation tends to reduce the moisture content in the surface layers, thereby aggravating the effects of salinity [Ayers (3)]. These factors, rather than a greater inherent sensitivity to salt, account for most observed failures of emergence on those saline soils which apparently can support the growth of established plants of the same crop.

Uhvits (95) discusses imbibitional and osmotic components of water absorption by alfalfa seed. Imbibition is not significantly affected by increased osmotic pressure of the medium but osmotic water uptake is. Seeds may remain viable in the imbibed state for long periods in saline soils. In connection with a field plot study [Bernstein *et al.* (15)], it was observed that sugar beet seed which failed to germinate and remained in moist, saline soil during an entire summer, germinated to produce normal seedlings the following fall when rains leached the salt from the vicinity of the seed. The mixed salts added to the soil ($NaCl + CaCl_2$) produced no apparent toxic effect even after this long period of exposure.

Kahn, Goss & Smith (64) recently reported that osmotic inhibition of the germination of lettuce seed by 0.15 *M* mannitol may be overcome by treatment of the seed with gibberellin. Further studies on the range of effectiveness of gibberellin treatment with respect to varying levels of osmotic inhibition and on the occurrence of this effect in other plant species would be necessary to evaluate the significance of this finding.

Vegetative growth and fruiting.—Although fruit yields tend in general to parallel declining vegetative growth as salinity increases, noteworthy exceptions to this relationship have been reported. Barley may decrease markedly in vegetative vigor as evidenced by shortening of stems and decreased straw weights while maintaining essentially full yields of grain [Ayers *et al.* (5)]. Similar effects have been observed on yields of seed cotton in relation to the vegetative growth of the cotton plant. Among the more tolerant forage crops, seed production is much less sensitive to salinity than yields of forage [Ayers *et al.* (5)]. Good fruit- and seed-production in vegetatively inhibited plants may be explained in terms of maintained assimilation rates and the favoring of fruiting associated with restricted vegetative growth.

Rice, on the other hand, may, at critical salinity levels, develop essentially normal straw yields but produce little or no grain [Kapp (65)]. This specific inhibition of flowering or fertilization, or both and fruiting suggests that rice may be particularly sensitive to moisture stress from the boot stage through the fertilization period.

In addition to inhibition of germination, early seedling growth stages may also be quite sensitive to salinity. Ayers *et al.* (5) found that moderate salinity caused only a retardation in emergence of wheat and barley seedlings. The plant in the four-leaf stage exhibited a marked sensitivity to an increase in salinity, however, even though levels did not exceed those which caused no yield depressions when applied at the boot stage. Rice seedlings also appear to be quite sensitive to salinity. Kapp (65) has found that tolerance of rice seedlings is low but increases appreciably in six-week-old plants. Del Valle & Babe (33) also report that rice increases in tolerance when 30-, 60-, and 90-day-old plants are compared. The authors have also observed that recent transplants of such crops as sweet potatoes, crucifiers, etc., are very sensitive to salinity. In these cases, root pruning associated

with transplanting increases the injury resulting from limited moisture availability in saline soils.

While plants adapted to salinity may increase in salt tolerance as they grow older, plants growing in nonsaline soils may become more sensitive to an abrupt increase in salinity as they increase in size, Thus, Iwaki (62) reports that rice becomes more susceptible to injury by brief two-day applications of sodium chloride solutions as the plants grow older. In general, development in a nonsaline medium with adequate moisture supply favors top growth. Excessive top growth may result in severe injury when moisture supply suddenly becomes limiting. For this reason, young plants generally adapt more readily to salinization than do older plants.

Because salinity retards vegetative growth, it may be expected to delay flowering. A delay in flower-bud formation of tomato and in anthesis of flax has been reported [Hayward & Long (55); Hayward & Spurr (59)]. The checking of vegetative growth by salinity may, however, hasten maturation and final harvest dates, especially of crops having indeterminate growth habits, such as potato, cotton, melons, etc. [Bernstein (8); Bernstein *et al.* (12)].

Decreases in fruit yield associated with salinity are usually the resultant of decreases in both fruit number and size [Bernstein (8); Bernstein *et al.* (9); Hayward & Long (56)]. Sweet corn is exceptional inasmuch as yields over a wide range reflect ear number entirely with essentially no change in ear weight. Ear number in turn is well correlated with vegetative growth of the crop [Bernstein (8)].

Salinity may, therefore, exert a variety of effects on plant development and yields depending on such factors as the nature of the crop (vegetative organs or fruit and seed), the determinacy of growth, the differential tolerance to salinity of the several stages of development, and other related or interacting factors.

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THE NATURALLY-OCCURRING AUXINS AND INHIBITORS[1,2]

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Several comprehensive reviews on various aspects of auxin physiology have previously appeared in this series (2, 31, 73, 144, 211); also other recent relevant reviews (72, 113, 157, 172, 201), and books (114, 222) are available.

This review is concerned with hormones controlling growth, i.e., the auxins, together with possible antiauxins and related inhibitors. Other developmental aspects controlled either by the same or other hormones, for example reproduction and fruit development, are not considered. The review covers evidence on the occurrence of the native auxins and inhibitors and their possible mode of action; degradation of the auxins is considered elsewhere in this volume.

The hormones controlling growth have become known generically as the auxins, i.e., growth regulators which induce cell enlargement at low concentrations. This definition, usually applied to cell enlargement in shoots, is the basis of most of the bio-assays used to detect auxins. Larsen (113) more rigidly defines this group of hormones as "substances which affect extension of the cell wall, accompanied by water uptake in the cell." Extension of the cell wall and water uptake are probably the aspects of growth most nearly related to the primary effect of auxins on the cell.

Many workers use the term "auxin" to refer to a single substance, 3-indoleacetic acid (IAA). To add to the confusion, IAA is the substance referred to in the earlier literature (and in current literature by some workers) as "heteroauxin." It would help considerably if workers using a specific chemical entity such as IAA would refer to it as such, and leave the term "auxin," which is physiologically defined, to be used as a generic term in physiological studies, where often the chemical composition of the active substance is not known.

The considerable volume of research on synthetic growth substances with auxinic properties has not led to an understanding of their mode of action, and it is not yet possible to construct a theory of growth which would fit all the facts revealed in this line of research. Work with the synthetic growth regulators, at present largely empirical, will be more rewarding when more is known on the mode of action of the natural auxins in plant tissues. Syn-

[1] The survey of literature pertaining to this review was concluded in July, 1957.

[2] The following abbreviations will be used: ATP, adenosine triphosphate; CoA, Coenzyme A; DNA, deoxypentose nucleic acid; EDTA, ethylenediamine tetraacetic acid; IAA, 3-indoleacetic acid; IAc, 3-indoleacetaldehyde; IAEt, 3-indole ethylacetate; IAN, 3-indoleacetonitrile; IAld, indole-3-aldehyde; IBA, indole-3-butyric acid; IPrA, indole-3-propionic acid; IPyA, 3-indolepyruvic acid; PNA, pentosenucleic acid; RNA, ribonucleic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid.

thetic growth regulators will not, therefore, be considered in this review, except incidentally.

Certain trends can be discerned in recent auxin research, due largely to the application of the powerful techniques of partition and adsorption chromatography. Firstly, there is a decline in the importance of auxins *a* and *b*, inevitable in view of the fact that they have never been detected in plant tissues and are not available to work with. Secondly, there is an increasing realisation of the widespread occurrence of various indole compounds, in particular IAA, in plant tissues, suggesting that these substances play a fundamental role in plant metabolism. Lastly, and perhaps the most important, there is a growing body of evidence on the occurrence in many plant tissues of as yet unidentified auxins or auxin complexes, some of them not giving typical indole colour reactions.

There has been a tendency in the past to consider that IAA is the major, if not the only plant auxin. Many papers are written from this point of view, especially papers dealing with the role of auxins in plant metabolism. This is perhaps unavoidable, since IAA has been until recently the only naturally-occurring auxin available in pure synthetic form for experimental purposes. Workers are becoming increasingly aware, however, that free IAA accounts for only a part of the auxin activity in plants, so that the central question now is, what proportion (if any) of the total auxin is IAA, and what are the other active compounds in plant extracts?

Fractionation and Bio-Assay of Extracts

Extraction.—Methods for identifying substances by determining rates of diffusion through agar are given in detail in *Methods of Plant Analysis* (113, pp. 614–21). More usually, standard chemical techniques of extraction and purification are employed. As the forms in which auxins occur in plants are largely unknown chemically (see discussion on complexes and precursors later), methods of extraction remain largely empirical. Some results with several standard organic solvents and water are reviewed by Larsen (113). Solvents such as ethanol or ether extract a range of "free" auxins, including many indole compounds (Tables I and II). Water extracts several as yet unidentified "auxin complexes" (Table III).

Purification.—Bio-assay of crude extracts has little meaning, because of the complicated mixtures of growth-promoting and growth-inhibiting substances which are usually present; consequently paper partition chromatography, paper electrophoresis and column chromatography are now widely employed as methods of purification before bio-assay. Gordon (73) and Larsen (113) list over 20 papers using these techniques. The majority of these workers used ether for extraction and examined the acid fraction, using paper chromatography under alkaline conditions (usually isopropyl alcohol, water, and ammonia). It is customary to run control chromatograms with synthetic compounds such as IAA and IAN to act as marker spots. Commercial IAA may need purification before chromatography (69). Some

of the chromatograms of the plant extracts are used to examine the position of the separated compounds in ultraviolet light (usually 2537 Å transmitted) and to examine them with various reagents giving colour reactions, usually reagents for indole compounds. Some of the chromatograms are cut up into suitable portions and the eluates from these portions examined for biological activity. Care should be taken with the papers under ultraviolet light, as many of the compounds are unstable and are rapidly destroyed by light and oxygen. Destruction of IAA by ring rupture can be practically complete on chromatographic paper under certain conditions (141). Papers not examined immediately should be stored in the dark under nitrogen.

Several critical surveys of the solvents used in chromatography of the indole compounds have been carried out (97, 149, 150, 178, 187). Nitsch shows that good separation of the majority of the acid auxins can be obtained by omitting ammonia and using only water and an alcohol, e.g., a mixture of isopropanol (80 parts) and water (20 parts). This is important, since it is now obvious that the presence of ammonia in the chromatography tanks is undesirable, as it can cause breakdown of labile substances (23, 89). Water alone gives a good separation of the three auxins IAA, IAN, and IAEt. Isobutanol:methanol:water::80:5:15 gives a good separation of IAA, indole-3-butyric acid, IAN, and IAEt. For better separation of the neutral auxins, Nitsch recommends a mixture of hexane (90 parts) and water (10 parts).

Nitsch's work clearly demonstrates that different extraction solvents and different chromatography solvents give different auxin pictures, both qualitatively and quantitatively.

Other solvents recently investigated are 50 per cent ethanol for IAN and isopropanol:water:acetic acid::100:10:2 for gramine (102).

Bitancourt (25, 26) uses ascending chromatography in water in an atmosphere saturated with acetic acid. The runs last only one hour, during which period the solvent front rises 10 cm. This technique avoids oxidation of IAA and other compounds, which can occur when the chromatograms run for long periods in organic solvents. Preliminary equilibration improves the quality of chromatography in water by giving relatively compact spots. The disadvantage of water appears to be that it does not separate IAA from inhibitors (49).

Methods of carrying out paper chromatography are legion. For example, an apparatus is described for carrying out paper chromatography and paper electrophoresis in a small space (14), or for carrying out large numbers of two-way chromatograms (56). A simple method for developing small chromatograms in glass tubes is described by Nitsch (149), adapted from (187).

Some workers have used a combination of column chromatography, one- and two-dimensional paper chromatography and electrophoresis, to yield better separation of active zones (61, 62, 124, 129, 130, 218, 219, 220).

An absorption technique using charcoal (55) offers a possible rapid method of obtaining the active water-soluble compounds which cannot be

directly extracted with organic solvents. This method may be especially valuable for hydrophilic indole conjugates. A further advantage is that it can be carried out at neutral pH under mild conditions.

Chemical determinations.—A variety of colour reactions can be carried out on chromatograms or eluates to determine the presence of indole compounds. Most of these colour reactions have the disadvantage that they are not as sensitive as bioassays. Among spray and dip reagents which have recently been intensively investigated are the Salkowski and Ehrlich reactions, with various modifications (58, 95, 97, 102, 130, 164, 178, 187, 226). All these authors list colour reactions and R_F values for many indole compounds, using a variety of solvents.

A fact worth noting, from an examination of these workers' results, is that many indole compounds do not give "typical" indole reactions of red and purple colours in the commonly used tests, so that claims for the existence of nonindole auxin systems based on failure of colour tests must be accepted with caution.

A number of workers have developed quantitative methods of determination of indole compounds on chromatograms (58, 67, 102, 128, 140, 148, 187, 213). It should be noted that quantitative methods, using colour tests, on unpurified plant extracts are probably highly inaccurate because of the interference of other compounds in the reactions. In particular, the Salkowski reaction seems to be affected by reducing substances such as phenols (165). If, however, the extract is chromatographed and the substance, e.g., IAA, eluted off the paper, accurate quantitative measurements can be obtained by a modified Salkowski reaction, using organic solvents (74).

Bio-assays.—The standard methods of bio-assay have been well reviewed lately (20, 21, 113, 114, 148, 150). The *Avena* curvature test is more difficult to perform than the straight-growth test and it gives more variable results, but it has the advantage for developmental studies that smaller amounts of auxin are needed to give a response. The paste curvature test, in which the active substance is applied in lanoline paste to one side of an intact coleoptile, has the advantage of using an intact organ, which can show up to 100 per cent inhibition or 100 per cent growth promotion. This advantage is lacking in almost all other test methods. The disadvantage of such an intact system is its lower sensitivity, due, no doubt, to its greater self-sufficiency.

Work continues on examination of the physiological factors responsible for differences in response obtained in the different tests (168).

The most widely used test for the examination of natural auxins is the *Avena* or wheat straight-growth test. Effects of many accessory food factors in this test have been examined, e.g., sugars (26, 150), vitamins (26), and metallic ions. Cobalt stimulates growth at very low concentrations in both *Avena* and pea (142, 199, 202). Manganese also stimulates growth, but at different concentrations from cobalt (199). The possible role of these trace metals in growth is discussed.

Factors such as effect of light, length of sections, age of grain, length of

seedlings used for test, etc., have been examined (26, 150, 209a), and largely agree with earlier work. Sections cut from long coleoptiles (*ca.* 30 mm.) are reported to be less variable in their response to added auxins than sections from shorter coleoptiles (108). They have the disadvantage, of course, that they respond much less than the shorter coleoptiles. The "intrinsic" growth rate of the sections is shown to be related to the growth rate of the coleoptile at the time the section is cut (13). These workers also explain the slight inhibition observed in sections in very low concentrations of IAA, by postulating an antagonism of IAA with the native auxin of the coleoptile.

As a result of his studies on some of the above factors, Nitsch (150) proposes a sensitised coleoptile test which increases sensitivity fortyfold; also a test using the first internode, increasing sensitivity two hundredfold. This makes the internode test as sensitive as the *Avena* curvature test. He rotates his internode sections on a wheel to get greater and straighter growth. This effect was previously reported for coleoptiles by Hancock & Barlow (75). The internode test has the advantage of not showing any response to amino acids, which may interfere with auxin assays of chromatograms of aqueous plant extracts when coleoptiles are used. Also, the test is less sensitive to inhibitors. On the other hand, the technique requires great care. All manipulations have to be carried out under green light, at a wavelength of about 546 mμ. Sucrose is used as a food factor, as it increases growth appreciably in the presence of IAA, but only to a very small extent in the controls.

The following generalizations on some of the points of technique of the straight-growth test may be helpful. These generalizations are based on results of experiments carried out by the present author under varying conditions over a period of several years.

1. Apparatus can be considerably simplified with experience. A dark, temperature-controlled room, although desirable, is not necessary. Oats can be grown in an incubator in red light, and the coleoptile test carried out in subdued daylight without loss of sensitivity.

2. Huskless oats are not necessary to obtain good coleoptiles or internodes. Ordinary oats can be grown in vermiculite. The internodes remain below the surface and can be exposed by shaking off the top, loose layer of vermiculite when required for use.

3. If germination is not good at 25°C., a lower temperature can be used (22 to 23°C.) without loss of sensitivity.

4. The year of harvesting the oats has a big effect on their sensitivity. A good summer in Great Britain (e.g., 1955) usually produces more sensitive oats than a bad summer. This fact probably explains different levels of sensitivity of American and British tests. Further variations in sensitivity can be attributable to atmospheric pollution (93). The ordinary straight-growth test (21), modified to use only 1 ml. solution in small dishes, with sucrose and a buffer, can be as sensitive as Nitsch's sensitised coleoptile test, but the lower limit of sensitivity in both tests is variable, and can be as much as 10 times higher than the reported minimum.

5. The author finds that usually sections in dishes show a higher response than sections rotated on a wheel. They can be kept straight by subjecting the dishes to a gentle, rocking motion. The most important factor affecting response in the assay is the speed with which the material can be handled. The author is much more skilled in assays carried out in dishes than on a wheel; probably the reverse is the case with operators who habitually use a wheel.

6. The lower limit of sensitivity in the internode test under optimum conditions in the author's laboratory is the same as that reported by Nitsch (10^{-9} gm./cc.), but is variable, and can be as much as 10 times higher.

Various sugars and salts used as accessory food factors alter growth rates considerably, and therefore have a big effect on growth/time relationships. They are able to hold the growth rate constant over a range of time intervals and IAA concentrations, IAA then being presumably the chief factor limiting the growth rate.

Other bio-assay techniques.—Several bio-assays make use of root tissues. Tests in which epidermal cell elongation is taken as a measure of root growth [e.g., (41)] have been criticised (206). Root inhibition continues to be used, but results must be interpreted with caution, because of the nonspecific nature of the response. Åberg (2) considers that growth stimulation of roots at low auxin concentrations has not been satisfactorily demonstrated, and probably does not exist. In five experiments carried out in 1949 using Moewus's technique (143), and cress seed kindly supplied by Dr. Moewus, the present author got an average maximum stimulation of 20 per cent at 5.7×10^{-13} *M* (18a). Nevertheless, growth stimulation in roots is certainly not consistent enough to be used as a means of measuring auxins, as has been recommended (151).

Other tests reported include petiole abscission and hypocotyl rooting [insensitive to inhibitors (131)], protoplasmic streaming in *Tradescantia* staminal hair cells [60 min. test (106)], hypocotyls of mung beans (111), and epinastic curvature of leaf petioles [useful in the tropics (35)].

Naturally-Occurring Auxins

IAA.—There is now a considerable body of evidence from chromatographic studies on the occurrence of IAA in a wide range of plant tissues. This was reviewed in 1954 (73) and 1955 (113), and more recent work has demonstrated the presence of IAA in further tissues, as shown in Table I.

It is interesting to note, however, that there are reports of workers being unable to demonstrate IAA in their extracts. These are also shown in the Table. In all these cases, either IAA was carefully looked for, or else sufficient material was used for it to be probably detected if it were present. It is unlikely that instability of the IAA during manipulation of the extracts could account for these results. Suggestions have been made that acid pH during extraction might destroy IAA, but it has been shown that there is no loss at pH <3.0 for a few hours (214). Also IAA is only slightly destroyed by autoclaving with *N* HCl at 15 lb. pressure for 15 min. (18a).

TABLE I

REPORTS OF PRESENCE OR ABSENCE OF IAA IN VARIOUS PLANT TISSUES

IAA Detected Tissue	Reference	IAA Not Detected Tissue	Reference
Bean and pea shoot and root	(16)	Maize coleptiles and roots	(91)
Sunflower shoots, maize roots	(16)	Apple leaves and fruits	(132, 133)
Cauliflower heads	(102)	Tobacco leaf, root and stem	(216, 217)
Brussel sprouts and cabbage	(102)	Cabbage	(89, 194)
Brussel sprouts and cabbage (inner leaves)	(130)	Brussel sprout and cabbage (green leaves)	(130)
Corn endosperm	(177)	Tomato fruits	(149)
Sunflower tumour tissues	(177)	Tomato roots	(38)
Strawberry achenes	(147)	*Topinambour* gall tissues	(110, 176)
Coconut milk	(149)	Oat, maize, barley coleoptiles	(69)
Citrus flowers	(188)	Pea, sunflower, cucumber, buckwheat hypocotyls	(69)
Corn kernels	(86)	Potato sprouts	(69)
Blackcurrant seeds, berries	(229)	Pea, tomato, cabbage stems	(69)
Gooseberry seeds, pericarps	(229)	Blackcurrant berries (immature)	(229)
Apple seeds	(229)	*Ustilago* tumours and healthy tissue (maize)	(209)
*Bamboo shoots	(109)	Soybean, spinach, barley	(213)
*Tobacco ovaries	(135)	Tomato leaf and stem tissue	(213)
*Tobacco pollen	(134)		
**Datura* ovule tumours	(175)		
*Various cultured tissues	(149)		
*Grapes	(149)		
*Sunflower shoots	(10)		
*Bean seedlings, pea roots	(10)		
*Tobacco leaves and stems	(104, 158) (159)		
**Hevea brasiliensis* leaves	(29)		
**Lunularia* thalli	(57)		
*Bean root nodules, cauliflower leaves	(228)		
*Maize kernels	(228)		
*Oat coleoptiles	(170)		

* R_F of active zone only.

Different results may be obtained at different stages in development of the tissues. Thus, IAA could be found in the colourless inner leaves of brussel sprout, but not in the outer, green leaves (130). Vlitos *et al.* (213, 216, 217) could only detect IAA after alkaline hydrolysis of tobacco leaf and stem tissues, pointing to the existence of an inactive IAA complex. IAA might be masked by the inhibitor scopoletin, which runs at the same R_F as IAA in isopropanol/NH_3. Methods for separating the two are given by Pavillard & Beauchamp (158, 159), who could thereby detect promoting activity in the IAA zone in tobacco. Kefford & Helms (104) could also

detect IAA in tobacco, their identification being based only on R_F of an active zone at the same position as IAA.

It should be noted that several reports of the occurrence of IAA (Table I) are based only on R_F values, in a single solvent mixture, of biologically active zones. These identifications must be accepted with caution. There are several reports of water-soluble auxins, which might separate partially into the ether fraction, with the same R_F in isopropanol/ammonia as IAA (Table III). Also Müller (145) reports an auxin much less active than IAA but having the same R_F and absorption spectrum. Raadts & Söding (170) suggest that the IAA which they find on chromatography of diffusates from *Avena* coleoptiles may be an artifact, coming from some "bound" auxin during the course of the experiment. The report (221) that a nonindolic auxin, having the same R_F as IAA, remained in extracts after all the IAA had been destroyed by IAA oxidase, has been modified; it has been found that IAA in the presence of plant extracts is only partially destroyed by IAA oxidase [von Guttenberg (221a)].

Thus, the presence of IAA in all plant tissues is not established. From physiological studies on roots, it is suggested (189) that IAA does not seem to be one of the hormones involved in aging (and, therefore, presumably in growth) in root cultures of tomato. Other workers (9) query whether IAA is one of the natural auxins of pea roots. Further evidence besides bioassay and single R_F values is needed before IAA can definitely be stated to be present.

To sum up the present position, the possibility cannot be dismissed that IAA may well not be the auxin operative in tissues at certain stages of development, especially since it often occurs only in small amounts relative to the total auxins present.

3-Indoleacetaldehyde.—IAc would be extremely difficult to detect by chromatographic methods because of its instability and rapid change to IAA (19). Reports on its occurrence in plants are as yet based only on indirect evidence of the occurrence of a neutral substance, sometimes showing some of the properties of an aldehyde, and convertible by various means, including aldehyde oxidase preparations, to an acid auxin, presumably IAA. This work is summarised in earlier reviews (73, 112). Much of it could have been dealing with the nitrile, which was not then known to occur in plants.

3-Indoleacetonitrile.—In addition to its original isolation (99), IAN is now reported to occur in tomatoes and grape seeds (149), brussel sprouts and cabbage (130), a variety of other *Brassica* species (61, 220), tumour tissues of *Nicotiana* (177), potato peelings (28), and tobacco ovaries (134, 135). It has usually (though not always) been identified by either the Salkowski reaction or the nitrite reaction as well as by R_F value and biological activity. Housley & Bentley (89) have demonstrated the release in large quantities of a substance, which they tentatively identify as IAN, from a water-soluble, ether-insoluble precursor. The identification is based on R_F value and a positive nitrite reaction. If correct, this is the first evidence of a substance

concerned in the biogenesis of IAN, and it is obviously important to investigate its properties further. 3-Indolecarboxylic acid and 3-indolealdehyde have both been demonstrated as possible degradation products of IAN (50, 194).

It is noteworthy that high activity can sometimes occur in the nitrile zone, although no colour reactions can be obtained (22, 38), even with heavily-loaded chromatograms. Activity in this region may be due to other unidentified substances (e.g., Table II and Table III, compound Z), so that the presence of the nitrile in such extracts must be demonstrated at least by evidence from multiple R_Fs and colour sprays, in addition to biological activity. More critical methods of chromatography, such as those adopted by Nitsch (149) for separation of the substances running near the solvent front, are probably of greater value than colour sprays, since bio-assays can detect much lower concentrations of active substances than are needed to give colour reactions. In any event, the final identification must rest on chemical isolation. This cannot be emphasized too strongly, as far too much reliance is being placed on suggested identifications from chromatographic results.

Methyl and ethyl 3-indoleacetates.—It has been suggested (83) that the original isolation in chemically pure form of IAEt from immature maize kernels (171) might be an artifact, as ethyl alcohol was used for the isolation. IAEt is now reported to occur in fair quantities in grape seeds and coconut milk (149), and in apple endosperm (197), though the latter identification has been queried (133). A substance running at the same position as IAEt, and well separated from IAN, has been demonstrated in grape seeds (148), corn endosperm, *Parthenocissus*, and tumours of tobacco and weeping willow (177), and also in corn kernels (86).

The methyl ester of IAA, which is biologically active, has been isolated from human urine (227), after refluxing a semipurified extract with methyl alcohol plus 1.5 per cent hydrochloric acid. These workers followed the same isolation procedure for auxin *a* as used by the original workers (107). The methyl ester results from IAA in the urine, and they conclude that only IAA occurs in their samples, and that no step in Kögl's original isolation could separate it or its ester from auxin *a*.

3-Indolepyruvic acid.—The presence of IPyA in maize endosperm has been reported recently, using methods of ammoniacal chromatography (186, 187). It is considered to be a growth hormone probably identical with accelerator α (16), and with other growth promoters described by Lexander (117) and Terpstra (195, 196). It is also reported in leaves of soybean and tobacco under short-day conditions (215). Pure synthetic IPyA has been prepared and examined (23), and this is shown to break down completely under conditions of ammoniacal chromatography. It is much more stable in acid, and can be chromatographed in an acetic acid:water mixture (1:3 v/v) with practically no breakdown except to slight traces of what is probably IAA. Ammoniacal silver nitrate is shown to be a very sensitive reagent for the

detection of IPyA, being reduced to a brown spot immediately in the cold.

The breakdown products of IPyA produced during ammoniacal chromatography can be resolved into a number of spots giving colours with the usual indole colour reagents. IAA can be readily detected, and, in addition, some of the other spots are biologically active. One of the active zones occurs approximately halfway between the starting line and IAA, in the same position as the substance reported by Stowe & Thimann to be IPyA. However, it gives none of the colour reactions typical of IPyA. Another active zone occurs just behind IAA. This has been tentatively identified as indole-3-glycollic acid (23). Fischer (61) reports the occurrence of indole-3-glycollic acid in various plants, but his substance (compound E) is running in front of IAA (see Table II). A sample of sodium indoleglycollate, kindly supplied to me by Professor Galston and Dr. Greensberg, Yale University, ran just behind IAA (18a) but also gave a faint spot in front of IAA, due either to a breakdown product or a contaminant. This second spot may be the spot observed by Fischer and may also account for the activity just in front of IAA on chromatograms of IPyA.

The biological properties of synthetic IPyA have also been examined (23). It has low, but real, activity in the *Avena* test, equivalent to approximately a 1 per cent conversion to IAA. Furthermore, it causes only inhibition of cress root growth, which means that accelerator α, which promotes root growth, is not IPyA, as was suggested (186). It has been shown that accelerator α promotes coleoptile growth at approximately the same concentration at which it promotes root growth (103). IPyA, at the concentration at which it promotes coleoptile growth (1 to 10 mg./l.), strongly inhibits root growth.

There are also two further, unidentified, active zones running ahead of IAA on ammoniacal chromatograms of synthetic IPyA.

Several other indole compounds have also been demonstrated in plant tissues by chromatographic techniques. These include 3-indolecarboxylic acid [probably by oxidation of IAN (50, 100)], 3-indolealdehyde (61, 100, 217), indole and N-methyltryptophan (188), 5-hydroxy-N,N-dimethyltryptamine (191), 5-hydroxytryptamine (36, 54, 163), 5-hydroxy-IAA (210), and skatole (141). Some of these compounds are probably concerned in the biogenesis or degradation of the indole compounds already considered [this point is discussed in greater detail in (18)]. All are inactive except 5-hydroxy-IAA, which has slight activity in the *Avena* straight-growth test (about 1/500 that of IAA) and 5-hydroxytryptamine, which inhibits root growth in a manner similar to indole propionic acid (68), although it is inactive in the *Avena* test up to 5×10^{-5} mg./l. (18a). The significance of the 5-hydroxyindoles in plant metabolism is so far not clear. Fish and co-workers (63), who have demonstrated the existence of N,N-dimethyltryptamine and oxide, and bufotenine and oxide in seeds and pods of *Piptadenia* species, suggest a new pathway of tryptophan metabolism via N,N-dimethyltryptamine to bufotenine and bufotenine oxide. Judging from studies on animals, bufo-

tenine and serotonin are evident products of tryptophan metabolism, maybe with 5-hydroxy-IAA as the end product, and it may be that similar pathways occur in plants.

Unidentified ether-soluble auxins.—In addition to substances which can be identified more or less certainly with known indole compounds, there are reports of a number of other, as yet unidentified, auxins. Some of these, reported since the last review in this series (73), are listed in Tables II and III. All the extracts listed here have been chromatographed in isopropanol /NH_3, and are, therefore, to some extent comparable. An attempt has been made to indicate activity of substances relative to the most active zone on the paper, which has arbitrarily been given an activity of 4. Thus, the figures 1, 2 or 3 denote that the substance is not the most active on the paper. These figures give some idea of whether the substance is only slightly active, or present in small quantities, or whether it is one of the major constituents of the extract.

In Table II, the majority of the substances have been located in ethereal extracts. In Table III, all the substances were originally obtained in aqueous or aqueous alcoholic extracts, as indicated, and some are ether-insoluble.

The substances have been grouped according to chromatographic behaviour, as far as the data permit and they can only be regarded as relative.

An examination of the tables brings out a number of points. In Table II, there appear to be at least three groups of substances with R_F lower than that of IAA in ammoniacal chromatography.

The first zone of activity runs close to the starting line. The α-accelerator reported by Bennet-Clark and co-workers (16, 102) would run in this group. Compound W is obtained from aqueous extracts of cabbage after treatment with heat and weak alkali (89), which rather suggests that it is not present in plant tissue before extraction. Evidence is presented by Housley & Bentley suggesting that W and the α-accelerator are the same substance or substances. In all the extracts examined, this zone of activity is only weak, so there will probably be considerable difficulty in getting a chemical identification. No colour reactions are reported by any of the workers.

The second group occurs at the same position on the chromatograms as the substance identified by Stowe & Thimann (186, 187) as IPyA. A number of workers have identified their compounds in this group as IPyA, mostly on evidence of R_F only. This could be misleading, as other indole compounds are found in this region. For example, 5-hydroxy-IAA, which may also occur in plants, runs at the same R_F in isopropanol/NH_3, has a positive Ehrlich reaction and similar biological activity as IPyA (somewhat less than 1 per cent of IAA), and would, therefore, be found in this group. Raadts & Söding decide that their active growth substance from *Avena* coleoptiles, running in this group, is not IPyA (170).

The third group appears to be present only in very small amounts or to have low activity. Tryptophan and indoleglycollic acid (both slightly active) and indolecarboxylic acid (inactive) would occur in this group. All these

TABLE II

UNIDENTIFIED ETHER-SOLUBLE AUXINS AND RELATED COMPOUNDS LOCATED IN PLANT EXTRACTS BY CHROMATOGRAPHIC METHODS

Symbol or Possible Identity	Material	Extract	Amount Found	Positive Colour Reaction	Reference
On or very close to the starting line					
W	Cabbage leaves, tomato roots	N	1	None	(38, 89)
—	Brussel sprouts	T	1	—	(130)
—	Various gall tissues	T	2	—	(149)
—	Apple leaves	A	1	—	(75)
IPyA	*Salix* tumour tissues	T	1	—	(25)
—	*Adiantum* apical meristems	T	3	—	(150)‡
—	Blackcurrant seeds	N	1	—	(228)*
Malus 1	Apple leaves, seeds	A	2	DMAB	(132)*‡
Half-way between starting line and IAA					
—	Cauliflower heads	A	1	Salk.	(102)
Not IPyA	*Avena* tips	$CHCl_3$+T	2	—	(183, 170)
—	Cabbage and tomato	A & N	1	None	(38, 89)
IPyA	*Nicotiana* tumour tissue	A & N	4	—	(25)
—	Cabbage, brussel sprout	T	1	None	(130)
—	Various gall tissues, tomato and grape fruit	T	2–3	—	(149)
IPyA	Potato peelings	A	1	—	(28)*
IPyA	Soybean, tobacco leaves	A	Present	DMAB	(215)
—	Blackcurrant, gooseberry and apple seeds	N, T, A	1–2	None	(228)*
Close behind IAA					
—	Grape fruits	T	1	—	(149)
—	Oat coleoptiles	A	1–3	—	(196)
—	Cabbage, brussel sprouts	T	1–2	Salk.	(130)
—	Cauliflower heads	A	—	DMAB	(226)
Between IAA and IAN					
—	Cauliflower heads	A	1	Salk.	(102)
Compound E	Cabbage, *Helleborus*, Spinach	EtOH	1	Nitrite	(61)
—	Swede roots	A	3	—	(88)
—	Maize kernels	T	4	Salk.	(187)
Malus 2	Apple leaves, seeds, fruits	A	4	None	(132)*‡
—	Various gall tissues	T	4	None	(149)
IBA	Potato peelings	A	4	—	(28)*
IPrA	Cabbage, brussel sprouts	T	A lot	Salk.	(130)
Ribes 2	Blackcurrant berries	A	4	Salk.	(229)*
—	Apple seeds, maize kernels	A	4	—	(228)*
Near solvent front					
—	Cauliflower heads	A	—	DMAB	(226)†
Malus 3	Apple leaves, seeds	A	3	DMAB	(132)*‡
S-indole compound	Cabbage	T	Inactive	Salk.	(194)

TABLE II (Continued)

Symbol or Possible Identity	Material	Extract	Amount Found	Positive Colour Reaction	Reference
Skatole ?	Cabbage	T	?	Salk.	(130)
IAld	*Helleborus*, cabbage	EtOH	Inactive	Nitrite	(61)
Ribes 3	Immature blackcurrant berries	N	Present	DMAB(?)	(229)*
—	Tobacco leaves, apices	A	Present	DMAB Salk.	(216)

All extracts prepared with ether, chromatographed in isopropanol/NH_3 and assayed by *Avena* coleoptile extension except where otherwise indicated.

* in *n*-butanol/NH_3.

† assayed on bean internodes.

‡ assayed on wheat.

A—acidic N—neutral T—total ether fraction DMAB-Ehrlich

TABLE III

UNKNOWN WATER-SOLUBLE AUXINS LOCATED IN PLANT EXTRACTS BY CHROMATOGRAPHY

Symbol	Material	Extract	Amount	Colour Reaction	Reference
Same position as IAA					
X	Tomato roots	H_2O, ether-insoluble	3	DMAB Yellow	(38)
IAN precursor	Cabbage	H_2O, ether-insoluble	3	None	(89)
—	Various brassicas	Aqueous EtOH*	4	None	(129)
—	Maize coleoptiles	H_2O, ether-insoluble	4	Atypical	(91)
Between IAA and IAN					
Y	Tomato roots	H_2O, ether-insoluble	1–2	None	(38)
—	Various brassicas	Aqueous EtOH*	4	None	(129)
B	Cabbage, maize seeds	EtOH†	1–2	Nitrite +ve	(61)
—	Maize coleoptiles	H_2O, ether-insoluble	4	None	(91)
Same position as IAN					
Z	Tomato roots	H_2O, ether-insoluble	2	None	(38)
—	Maize roots	H_2O, ether-insoluble	—	—	(91)
—	Various brassicas	Aqueous EtOH*	4	None	(129)
G‡	Cabbage	H_2O, ether-insoluble	2	Nitrite +ve	(61, 219, 220)

* column chromatography on Al_2O_3

† electrophoresis

‡ slower than IAN in electrophoresis

zones need re-examination using more refined chromatographic techniques to get more critical separation.

A number of indole compounds, some in fairly large quantities or with high activity, have been located between IAA and the solvent front. Some have been tentatively identified, e.g., indole-3-glycollic acid [Compound E (61), but see also the discussion on IPyA], indole-3-butyric acid [IBA-(28)], indole-3-propionic acid (IPrA) and indole-3-aldehyde [IAld- (61)], and skatole (130). These are probably degradation products of IAA or its precursors. An interesting compound, *Malus* 2, shows considerable activity, yet Luckwill (132) reports that he has been unable to obtain any indole colour reactions with this zone. He concludes that he must be dealing with a non-indolic auxin.

In addition to those reported in the table, various active zones have been located by Nitsch (149), using a hexane and water mixture to get good separation of substances running in front of IAA. Some of these zones are located between IAA and IAN, and some in front of IAN.

Nitsch has located seven auxins by chromatography in ammoniacal isopropanol, and five by chromatography in water. There seems to be a whole chemical "family" of interrelated growth substances. Also, a highly-active auxin of R_F 0.05 in water is reported in extracts of *Avena* coleoptiles and willow tumour tissue (177).

The S-containing indole compound which runs at approximately the same position as IAN (194) is of interest, although it is reported to be inactive. Various S-containing analogues of the indoleacetic acids have been tested and found to act as auxins (105).

The indole substance (not IAEt) running at the same position as IAN (216, 217) can be separated from IAN in other solvents, and is active over a range of tests. It is possible that reports of activity in acid solutions previously ascribed to IAN [e.g., (16, 102)] may be due to this substance.

Two conclusions can be drawn from Table II. One is that there are certainly some unidentified auxins which appear to be nonindolic (e.g., *Malus* 2). The other is that there are several as yet unidentified indolic compounds. Some of these may be concerned in the biogenesis or degradation of tryptophan, as part of basic cell metabolism, with no significance as hormones affecting growth and development. Still others may be artefacts, produced during extraction or chromatography by breakdown of unstable indole compounds such as IPyA and indoleglycollic acid. Whether any of them are auxins in their own right remains to be shown by further developmental studies.

Unknown water-soluble auxins.—Hitherto, most work has been carried out on ethereal fractions. Recent work has extended to the aqueous ether-insoluble extracts and it has revealed the interesting fact that they are highly active (Table III); in many instances they are considerably more active than the ether extracts, which would contain all the known auxins. A further interesting fact is that, although present in large enough quantities, most

of the compounds present in the extracts do not give characteristic indole colour reactions, which has led various authors to postulate a nonindole system of auxins in these plants (38, 130).

The first group in the Table run at the same position as IAA in ammoniacal isopropanol. The precursor of IAN, demonstrated in cabbage (89), runs at this position though it runs behind IAA in ammoniacal *n*-butanol. Compounds X and Y both act as precursors of Z and are interconvertible (38). It is possible that the same system is present in cabbage, although masked by the nitrile-producing system.

The question of the identity of these substances, present in the extracts in such large amounts, is obviously of great interest. They could denote either the presence of a nonindole system, or there might be some fairly simple change of the indole molecule, e.g., the addition of hydroxy groups, which would confer water-solubility. Hydroxy-indoleacetic acids do not always give characteristic indole colour reactions (187, 226). If the active points on the indole nucleus required to react in the various colour reactions are blocked with other substituents then a characteristic colour reaction will not be obtained. Alternatively, they may be auxin complexes not giving the characteristic indole colour reactions, but releasing the indole auxins, e.g., IAA or IAN, under suitable conditions. IAN is released under conditions of mild heat and alkalinity from a precursor which could be an auxin complex, and there is evidence also that IAA is released (89). Linser & Maschek also suggest that they may be dealing with a highly-active IAA complex (129).

The interconvertibility of the active compounds in tomato (38) suggests that we may perhaps be dealing with a system of monomeric-dimeric forms. For example, it has been stated that the dimer, N, N^1-diindolyl-3-, 3-diacetic acid, a possible intermediate in the oxidation of IAA in the Salkowski reaction, is as active as IAA in certain tests (87).

It is quite clear from the results in Table III that the auxin compounds in plants are not a series of simple indole compounds, yet in view of the widespread occurrence of biologically-active indole compounds in plant extracts, it would be more reasonable to suppose that the new auxin compounds are related to the indole substances in some way, rather than constituting a separate biologically-active system. Not enough information is available yet to judge on this point.

Unknown Auxin Complexes and Precursors

Various theories on the physiological significance of the bound auxins, auxin complexes, and auxin precursors in plant tissues have been discussed by earlier reviewers [e.g., (72, 73, 113, 114)]. Full clarification of the situation will not be achieved without chemical knowledge of the substances concerned. Probably the most valuable criterion at the moment is the physiological one of whether the compound in question is an immobilized form of auxin, e.g., the bound auxins which act as storage reserves in seeds (73 p. 359, 82), or whether it is itself actively concerned in the growth process.

Some workers consider (1, 73 p. 342) that bound auxins are inactive and that such complexes do not function as auxin precursors *in vivo*. Veldstra (212) suggests that various auxin complexes may be a combination of active auxin with "sites of loss" in the tissues, due for example to unspecific adsorption, detoxification, or tissue storage.

Nevertheless, there is early evidence of the existence of water-soluble, relatively ether-insoluble auxin complexes which act as precursors of IAA, usually by treatment with acid or alkali. These substances often appear in large quantities relative to the "free" ether-extractable auxins present in the tissues [e.g., (11, 24, 156)]. Recently, water-soluble precursors in the oat coleoptile have been examined using chromatographic techniques (170, 183). On treating with acid, an ether-soluble substance of the α-accelerator group (Table II) is obtained. On treating with alkali, this and a substance running at the IAA position is obtained.

Terpstra (196) suggests that there is very little free auxin in coleoptile tips, and that all the activity is present as a complex which is extractable with water and is ether-insoluble. The complex yields IAA and possibly other free auxins on treatment with alkali and extraction with ether. The same seems to happen with the water-soluble, ether-insoluble compounds examined by Housley & Bentley (89). A neutral extraction similar to Söding & Raadts' diffusion into water is probably the most suitable for obtaining these complexes, and extraction of dried tissue with dry ether the most suitable for obtaining any free auxin which may be present (probably very little). It would appear that bound and free auxin are in a dynamic state in the plant. The interesting question is to determine their relative roles. The report that there is much free auxin (extractable in the first 15 min.) in crown gall tissue, whereas most of the auxin in normal tissues is in a bound state, is of interest here [(110) discussion by Morel].

A water-soluble precursor which yields IAA by ether extraction after mild heat treatment is reported to be present in a number of plants, especially maize seeds (219). This is perhaps comparable with the precursor which yields IAN on mild heat treatment (89). Also Bonde (30) reports an acidic precursor of a neutral growth substance in cabbage. The precursor is water-soluble rather than ether-soluble. It is possible that these substances are all identical, and that the nitrile is produced in cabbage rather than the acid because of interaction with a third substance. The production by heat treatment of IAA, and possibly also IAN, from a water-soluble precursor present in coconut milk has been demonstrated by partition chromatographic methods (149).

What can be concluded on the chemical constitution of these compounds? Inactive precursors could possibly be compounds such as tryptophan or tryptamine which may be concerned in the biogenesis of IAA. It has been shown, however, that in the higher plants only very small amounts of auxin can be obtained from available tryptophan, much less than has actually been demonstrated in tissue extracts (185, 231). Alkaline saponification (*N* NaOH,

15 lb. pressure, 15 min.) of tryptophan does not yield extractable auxins, yet under these conditions highly active indole compounds, possibly obtained from indole complexes, occur in plant extracts from which all the ether-soluble indole hormones have previously been removed (18a). These facts speak against tryptophan acting as an immediate precursor of the indole hormones.

Andreae and his colleagues (4, 5, 69) have investigated the compound formed when IAA is fed to a wide variety of tissues, and have identified it as 3-indoleacetyl aspartic acid, which is water-soluble and only sparingly ether-soluble. This compound or similar indole conjugates, if they occur naturally, could account for some of the results in Tables II and III. It has been suggested that such compounds are detoxification products of IAA,—for example, indoleacetyl-L-glutamine appears to be a detoxification product in humans (96). Other synthetic indoleacetyl peptides with interesting biological properties have been examined by Jerchel & Staab-Müller (98).

There is evidence (reviewed in 113) that some bound auxins are probably auxin-protein complexes. Certainly, auxins can be obtained from tryptophan-containing proteins by relatively drastic means (e.g., enzymatic hydrolysis, prolonged boiling with alkali) yet, as Leopold points out (114), auxin production in the plant is not generally associated with protein breakdown, but with protein synthesis. The formation of a reversible auxin-protein complex *in vitro*, via the sulphhydryl groups in an enzyme preparation of pea shoots, without the intervention of such intermediate metabolic systems as coenzyme A (CoA) or ATP, is claimed by Marrè (136). He thinks that this complex probably plays a large part in the physiological action of auxin, though his evidence for this is scanty.

Galston (66, 179) has shown that the IAA bound to pea protein *in vivo* is not destroyed by IAA oxidase, and could therefore act as a store from which an IAA complex, resembling Andreae's indoleacetyl-aspartic acid, is split off by the action of CoA *in vitro*. The theory earlier put forward that auxins may form thioesters with CoA (115) is now doubted (116).

It seems reasonable to suppose that some of the results in Table III can be explained by postulating that IAA and IAN, and possibly also other hormones, are bound to some molecule conferring water-solubility and in some cases interfering with the development of characteristic indole colours by the indole nucleus. Carefully-controlled hydrolysis experiments are needed to give a lead on the nature of the binding substrate. From the chromatographic behaviour of the substances reported in Table III, it is doubtful that they are protein conjugates, but more probably substances of much lower molecular weight. If they are complexes of auxin bound to some other molecule, then the fact that the auxin cannot be eluted by exhaustive extraction, e.g., with ether, suggests that the binding forces are rather strong, i.e., of the nature of true chemical bonds rather than electrostatic or van der Waals forces.

The existence of a physiologically-active natural auxin complex is sup-

ported by various other observations. The results of a theoretical analysis of growth-rates of *Avena* coleoptiles in the presence of various concentrations of IAA and other auxins suggest that the growth rate is proportional to the amount of complex formed (33). Similarly, Yamaki (230) concludes that CO_2 fixation in the oat coleoptile due to applied IAA occurs only when a physiologically-active IAA-complex is formed. From another angle, evidence has been presented that the easily extractable "free" auxin of the oat coleoptile is not the fraction active in cell elongation (64).

From the above reviewed papers, it appears likely that both applied and natural IAA may not be active at the cellular level until it is first transformed into some other active agent or complex. If this is so, it explains why it has not been possible to demonstrate an effect of IAA on numerous enzymes *in vitro*, although such effects are easily demonstrable *in vivo* (201, Table I).

It is difficult to draw rigid conclusions from the above survey of the naturally-occurring auxins, but several points can be made. Firstly, it seems fairly clear that IAA is not the auxin physiologically active in normal growth. It does occur free in many tissues, but only in small amounts compared with the (relatively) ether-insoluble auxins, which are possibly the physiologically active forms. The evidence is fairly strong that IAA and other indole compounds are produced by hydrolysis of these other auxins, which are therefore complexes of some kind.

Secondly, the role of the indole conjugates, which may be related to these complexes, needs further investigation. Are they purely detoxification products, or do they have a wider physiological role?

Thirdly, the possibility of the existence of nonindolic auxins cannot be dismissed. Whether these are true auxins, or whether they are compounds acting together with the indole auxins, after the manner of the giberellins or kinetin-like factors, as part of a multifactorial process remains to be investigated.

Further work on the identification of the unknown auxins discussed above is urgently needed, since knowledge of the mechanism of action of the auxins and the pathways of biosynthesis and degradation, involves in the last analysis a knowledge of the chemical nature of the substances concerned. Such studies are essential for an understanding of the basic cellular metabolism of plants.

Inhibitors and Antiauxins

Following the successful exploitation of the concept of antimetabolites in animal physiology, much attention has been given to detecting systems of antiauxins or auxin antagonists in plant tissues.

An antiauxin is defined as a substance "which inhibits competitively the action of auxins" (207), though such a competitive inhibition is very difficult to prove, and if it does occur, is probably found at many stages in the uptake, transport, or action of the substances in the tissues.

Auxin antagonists are defined as substances which are themselves inactive, but which counteract externally-added auxin.

Much more evidence has accrued on the antiauxin effects of applied synthetic compounds, than on the existence of natural antiauxins. This is only to be expected, considering the difficulties of investigating the natural growth regulators, and the ease of applying synthetic substances to tissues. The difficulties of the latter approach lie in the interpretations, and until we know more about the chemistry of the cell, it is clear that this type of approach must remain largely empirical.

Most of the work on these substances has been carried out on roots, and recent reviews include those of Torrey (206) and Åberg (2).

Among others, Burström has critically examined the concepts of antiauxin and auxin in connection with cell elongation in wheat roots (43, 45) and has shown that synthetic growth regulators may exhibit both auxin and antiauxin activity, depending on which part of the growth process they affect. To call a growth-promoting action in roots an antiauxin effect may well be a fallacy. An antiauxin should not only be defined by its ability to cause increase in root elongation, but also by a corresponding decrease in coleoptile extension. Burström (44) suggests that the term "antiauxin" should not be used at all in roots. Studies on cell elongation in roots have led to the introduction of a new term "root auxins" (76, 77). These compounds are active as root growth promoters, and also counteract external auxins.

From studies on the growth stimulating effects of IAA and certain antiauxins on roots, Audus & Das (8) suggest that these substances exert identical physiological actions in the same growth system. Åberg (2), however, does not accept the experimental evidence of stimulation of intact roots by auxins, and considers that the classical concept of a supraoptimal endogenous auxin concentration in roots is correct. Further work on the nature of the endogenous auxins of roots is clearly needed. The demonstration of natural auxins which are stimulatory to both root and shoot growth [e.g., (89, 91)] is highly significant in this context.

Åberg has tested many compounds which have antiauxin properties in roots (1) and considers that the hypothesis of a competitive antagonism still best fits the facts as known. On the other hand, the effect of a range of synthetic auxin antagonists and antiauxins on metabolism has been examined by Marrè & Dugnani (138), who suggest that they act by inactivating some fundamental dehydrogenase system, possibly by blocking SH groups, even in the absence of auxin.

Thimann points out that the competitive nature of the inhibition caused by so-called antiauxins may be only apparent (198). The observed effects may be better explained, as may also a number of synergistic effects, as a "natural consequence of the interaction of a group of limiting factors in controlling a multifactor process." Much care is needed in interpreting the

effects of mixtures of auxins and antiauxins applied externally, as there is evidence [e.g., (173)] that biological antagonisms in such mixtures may be exerted on the absorption rather than on the auxin-controlled growth process itself.

Naturally-occurring inhibitors, although not necessarily having antiauxin functions, are obviously very widespread. Earlier reports are summarised by Evenari (59). Later reports include an inhibitor of shoot elongation and lateral root initiation isolated from pea roots (92), which may be identical with an inhibitor of lateral root initiation produced by root tips (205). Also reported are a stable, water-soluble inhibitor from dormant peach flower buds (84), and a neutral inhibitor in grape internodes and buds (184). All these inhibitors are ether-soluble. A neutral water-soluble inhibitor in cotyledons of *Sinapis alba* seedlings, which controls development of the axillary buds, is also reported (162).

Some workers have demonstrated the presence of inhibitors by paper partition chromatography. Recent reports include studies on various gall and tumour tissues (25, 27, 149), potato (28), cabbage (89, 130), tomato roots (38), *Avena* coleoptiles (183), citrus flowers (188), seedling sunflower shoots and pea roots (10), two germination inhibitors isolated from lettuce and xanthium seeds (223, 224), apple leaves (132), blackcurrant berries (229), cauliflower leaves, gooseberries, root nodules, and dwarf bean seedlings (228).

Sometimes an inhibiting zone occurs at approximately R_F 0.9 in ammoniacal isopropanol (10). More usually, inhibitors are found in a broad zone running just in front of IAA, and called the β-inhibitor zone (16, 102).

We still possess virtually no knowledge of the physiological role of the β-inhibitor zone. An attempt to elucidate the chemical nature of substances responsible for inhibition in this zone in the acid ether fraction of potato peelings showed that it has not been possible to associate the inhibition with any one compound (194). Experiments carried out by the present author and colleagues at Manchester on extracts of the β-inhibitor zone kindly supplied by Dr. Taylor showed that inhibition in *Avena* straight-growth assays was a toxic effect. This toxicity was caused by a mixture of at least six compounds, possibly fatty acids, at greater than physiological concentration. One of them was identified by Dr. Taylor as azelaic acid (heptane 1:7 dicarboxylic acid). The coleoptile sections showed loss of turgidity, suggesting an effect on the permeability of the membranes. The damage was irreversible after 3 hr. in inhibitor solutions, and there was no evidence of a truly competitive effect when the inhibitors were tested together with IAA (as would be expected from an antiauxin). Luckwill (132), who shows inhibition in the inhibitor β zone in the coleoptile test in extracts of apple leaves, gets no inhibition in this zone when tested by the bean rooting and *Coleus* abscission tests. These tests might reasonably be expected, by the nature of the technique, to be less affected by fatty acids.

Previous suggestions that inhibitors played a physiological role in the

resting period of the potato have now been modified by the observation that these substances occur largely in the peel and not in the internal tissues (81). By the techniques used in these studies, the inhibitors could be toxic compounds of no physiological significance. Also, Burton (49) has been unable to find any relationship between inhibitor content and onset of sprouting in potatoes. Burton thinks that the net activity of the substance in the inhibiting zone on paper chromatograms reflects the oxidation-reduction status of the tuber.

Nevertheless, some evidence does exist of regulatory functions of inhibiting substances. Extracts of plum and apple woody shoots and apple leaves contain inhibitors which are nontoxic to *Avena* coleoptile sections; the sections recover from the inhibition when removed from the solutions (12, 13, 132). Kefford (102) has shown a possible relationship between inhibition of lateral buds and concentration of the β-inhibitor, which he thinks is an indole or phenol compound. The β-inhibitor in blackcurrant berries has a dosage /response curve identical with coumarin (228).

There have been some interesting reports recently of interactions and interrelationships between growth-promoting and growth-inhibiting effects. For example, the growth promoter called the α-accelerator has been shown to have an inhibiting effect on the *Avena* coleoptile after about 10 hr., both alone and mixed with IAA, even while still in physiological concentrations. Also, the β-inhibitor has been shown to promote growth in the first 5 hr. (103). It is possible that the compounds present in these zones were not completely separated. Such effects could be obtained from mixtures of substances entering the tissues at different speeds.

Further evidence of interactions comes from physiological studies on promoting-inhibiting systems acting *in vivo* in the tissues. Libbert, in a series of researches (118 to 121), has produced evidence of an inhibitor-precursor present in roots of pea plants. This interacts with IAA to give an ether-soluble, neutral inhibitor, which he believes is the correlative inhibitor of lateral bud development postulated by Snow. Treatment with weak acid or alkali releases an acid auxin, probably IAA, from the inhibitor. This suggests that the inhibitor may be one of the IAA precursors or complexes already considered, although Libbert suggests that it might be an unsaturated lactone. Since the inhibitor-precursor is synthesized by the roots, it might tie up with the inhibitory growth-regulator of roots, postulated by other workers (7, 9, 189, 190), although Audus (7) later modified his suggestion (8).

In later researches on the physiology of lateral root initiation (122, 123), Libbert proposes that his correlative inhibition precursor is itself an inhibitor of lateral root production. The substance is soluble in ether, water and benzene, is alkali-labile, acid-stable and is destroyed by H_2O_2. Ether extracts of his pea roots also contain an acid auxin, a neutral aldehyde-like compound (IAc?) and another weak, benzene-insoluble inhibitor.

Torrey (205) also reports production of an inhibitor of lateral root forma-

tion by pea root tips. The inhibitor is ether-soluble. He interprets lateral root formation as the result of a balance between chemical factors moving from the cotyledons to the root tip, and this inhibitor. Further work on the chemical constitution of these inhibitors is obviously needed.

Pohl's root-growth inhibitor isolated from maize scutellums may also fit in here (166, 167). This has a molecular weight of the same order as the antiauxin and auxin precursor of the oat coleoptile (65, 182 p. 48), although Pohl does not consider that his inhibitor is an antiauxin or auxin antagonist, since it does not restore growth promotion in roots which are inhibited by IAA. Pohl suggests that his inhibitor might be 3-indoleacetaldehyde. It should be quite feasible to test this hypothesis. It is interesting that indoleacetaldehyde has been postulated as an inhibitor in the *Avena* straight-growth test, on quite other evidence (19).

The suggestion that germination of lettuce seeds is prevented by inhibitors in the dry seeds (169) is weakened by the fact that considerable amounts of inhibitors are demonstrated in germinating seeds, notably after 48 hr. germination.

A naturally-occurring factor which may rank as an antiauxin is the factor which accelerates abscission of intact leaves, and which can counteract the retardation of abscission brought about by IAA (155); also the substance which inhibits cellular proliferation induced by IAA (192). Similarly, a water-soluble, ether-insoluble factor in the difusate from germinating *Digitalis* seeds has been shown to increase root growth of a number of species, and may have antiauxin properties (174). There is not sufficient evidence as yet to determine the physiological significance of these substances.

Thus, although some of the natural inhibitors examined are exerting a nonphysiological, possibly toxic, effect on tissues, there is also evidence of regulatory functions of inhibitors. There is an increasing body of evidence from tissue culture work that regulation of otherwise uncontrolled growth is achieved by growth inhibitors which accumulate in normal tissues. Normal growth correlations probably result from a system of interactions which are governed not only by the relative proportions of promoting and inhibiting substances, but also by the general nutrient level.

Little further advance can be expected in this field until these various naturally-occurring inhibitors have been chemically identified and made available for physiological studies.

Several unsaturated lactones which occur naturally and act as inhibitors have been chemically identified. Coumarin, for example, inhibits seed germination and root growth. This may well be by reason of its ability to ring-open to give *cis*-cinnamic acid, which is active as an auxin, as coumarin also causes cell elongation in *Avena* coleoptiles [(200) and author's unpublished observations]. Several coumarin derivatives occur naturally and have inhibitory properties (71). The behaviour of a range of coumarins and related compounds in filter-paper chromatography has been investigated by Swain (193). Scopoletin (7-hydroxy-6-methoxy-coumarin) has been isolated from

a number of species of flowering plants (listed in 70) and recently confirmed in potato (194). It has been suggested that the inhibitory effects of coumarin derivatives are due to the inactivation of sulfhydryl-containing enzyme systems, on the basis of the known reaction of thiols with α, β-unsaturated lactones (51). This explanation of inhibitor action assumes that the primary auxin effect is exerted via a thiol compound such as CoA. Such an effect would be a true competitive inhibition.

Mechanism of Action

Considerable work has been done on auxin action at the level of measurable growth, especially studies on the extension of isolated organs, such as *Avena* coleoptile sections. General conclusions of recent work are that the effect of IAA is primarily on the cell wall, thus bringing the wheel full circle to the early conclusions on the mechanism of auxin action (85). Bonner and colleagues (31) conclude that the first result of auxin-induced growth is increased uptake of water, and that the cell wall, rather than the cell membranes, is the primary barrier to water movement (153, 154). They suggest that auxin acts by bringing about a relaxation of cell-wall pressure (53).

A possible mechanism for this may be via the pectic substances of the cell wall. Incorporation of the carbon atoms of methyl-labelled methionine or of glucose into the hot-water soluble fraction of the cell wall (probably pectic substances) is enhanced in the presence of IAA, even when IAA-induced growth cannot take place. Bonner and colleagues consider that auxin-induced water uptake is not due to the effect of auxin on a metabolically-controlled component of internal diffusion-pressure deficit, or to effects of the auxin on the osmotic concentration of the tissues (34, 152). Thimann & Samuel (203) have studied the uptake of tritiated water by potato discs, and also conclude that the mechanism of cell enlargement is through a decrease of the mechanical coherence of the cell wall; they postulate that this is achieved by a metabolic process, resulting in the entry of water under osmotic forces.

Similar conclusions are reached by Buffel & Carlier (40). These workers have demonstrated with potato tissues that auxins cause a shift in cell-wall composition with a rapid build-up of pectic substances as opposed to cellulose content. Since the pectic substances are more highly hydrated and less crystallizable than the cellulose, they suggest that this change may be the immediate cause of the increased extensibility of the walls.

An increase in pectin-methyl esterase, an enzyme participating in the metabolism of pectic compounds, has been demonstrated in tobacco pith parenchyma cells in tissue culture, when the growth is stimulated by IAA (39, 146). This work is an example of the use of *in vitro* cultures for the study of enzymatic mechanisms involved in cell growth, a technique which holds promise of being very valuable.

As a converse of increased pectic synthesis during growth promotion due to IAA, it has been shown that concentrations of IAA which inhibit root

growth enhance synthesis of α-cellulose in the roots, probably leading to increased rigidity of cell walls (160).

From a study of cell extension in roots, Burström (42, 43) also concludes that it is the condition of the cell wall which generally limits growth. He postulates two stages of cell extension, and discusses the interaction of Ca, N, and auxins on these stages. Kögl & Mulder (107a) postulate a calcium deposition in the pectin of the cell wall in roots under the influence of IAA, thus changing the permeability.

Changes in permeability of inner protoplasmic membranes under the influence of IAA are also invoked, such changes probably making reserves available for metabolism or controlling distribution of nutrients (3, 6).

Although the evidence for a primary effect of IAA on the cell wall is strong, there is also considerable evidence for the participation of IAA in basic intracellular metabolism. These effects may, of course, be secondary consequences of hormone action. For example, Marrè and colleagues postulate a close link between the action of growth hormones and the oxidation-reduction equilibrium of glutathione, via ascorbic acid metabolism (137, 139, 204). A significant interaction between ascorbic acid and IAA during growth has been demonstrated by Chinoy *et al.* (52). Such a link would enable auxins to have a wide influence on the regulation of metabolism, and hence of growth.

An intimate connection between auxins and nucleic acid metabolism has been established, particularly through the work of Skoog and colleagues (180). They have studied the effect of IAA on nucleic acid levels in various tissues, e.g., tobacco pith tissue in culture, corn roots, and the lateral buds of *Tradescantia*. From their results, they conclude that marked IAA-induced increases in pentose nucleic acid are associated with cell enlargement, and marked increases in deoxypentose nucleic acid with cell division. They propose that the auxin level affects the DNA/PNA rates, which in turn influences the relative rates of cell multiplication and cell enlargement. Other purines may also be concerned. Kinetin (6-furfuryl-aminopurine) is a cell division factor on tobacco wound callus tissue, but only in the presence of IAA (94). Kinetin (a sample kindly supplied to me by Professor F. Skoog) is slightly active in the *Avena* straight-growth test, probably by reason of some synergistic interaction with the native auxin of the coleoptile. Thimann (198) suggests that maybe the auxins and the purines react at the same enzyme system. An *in vitro* stimulating effect of IAA on acid phosphatase activity in extracts of potato and maize roots has been demonstrated by Turian (208), who postulates a link between this effect and RNA-protein synthesis.

Another interesting set of interactions are those of the auxins with the giberellins. For example, the effect of giberellic acid appears to be mediated through IAA (37). The giberellins were reviewed in Volume 8 of this journal, and their interactions with auxins discussed (188a).

Further interrelationships have been shown between IAA and calcium (45, 46) in root cell elongation. It has been suggested that IAA might act by removing calcium from the cell wall by some chelation mechanism (15).

Heath also has suggested that IAA has a chelating action (78, 79), though there is no chemical evidence for this. If IAA acts as a chelate, then whatever is chelated becomes the real growth regulator, IAA being degraded to a regulator of the cation supply. A similar role to an auxin is envisaged for EDTA, a recognised chelating agent, in its promoting effect on coleoptile growth (78, 79) and also in its effect on lupin hypocotyls, where it is suggested that it forms a chelate with the calcium of the cell wall, thus enabling the cell wall to expand (225). The effect of EDTA on elongating coleoptiles is, however, extremely small and in no way comparable with a true hormone effect such as IAA produces (18a). It has been suggested that the slight activity of EDTA, which is similar to that obtained with other organic compounds not having hormonal activity, is attributable to a slight toxic stimulation of the coleoptile tissues (60). Burström (48) has shown that the EDTA-action on roots in no respect resembles an auxin effect. In any event, in view of the fact that many cations, e.g., Mn, Co, K, and Ca, have been shown to affect the growth process, it is not surprising that chelating agents such as EDTA, by altering the balance of available cations, should also affect growth either alone or in interaction with IAA.

Finally, attempts have been made to determine the molecular site of action in the cell by various mathematical techniques. Recent kinetic studies (32, 33) have supported Skoog's two-point attachment theory (181) though there has been some criticism of the techniques used to achieve a constant growth rate (17, 90). Burström & Hansen (47) point out that, in roots at least, an apparently constant rate of growth is, in reality, an integral of a number of different growth rates.

Another approach has been adopted by Linser and co-workers. By examining numerous mixtures of different growth promoters and inhibitors in different concentrations and applying a mathematical analysis to the results, they have worked out a method whereby they can determine whether a growth-promoting or inhibiting molecule is occupying a particular site in the system concerned with cell enlargement (101, 124a to 127). They also conclude, from their results, that more than one kind of growth reaction is involved. It seems that methods of approach such as these are over-simplified and unlikely to lead to a knowledge of the basic hormone action.

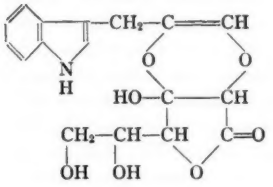
Thus, a large amount of work has been carried out on the mechanism of auxin action, but no firm conclusion, even as to the site of action in the cell, can be reached. It is comforting to find that the animal hormone physiologists are in much the same position (80). Work with animal hormones is tending to the concept that hormone action involves a primary reaction, probably affecting cell permeability to metabolites, which again is a return to the pioneer views. In the same way, thoughts on the action of plant hormones have returned to the original concept of an effect on the cell wall, though modern thought is couched in more elegant terms, invoking an effect via aspects of cell metabolism.

A major difficulty in hormone research is to recognise when observed physiological effects are secondary consequences of a primary hormone

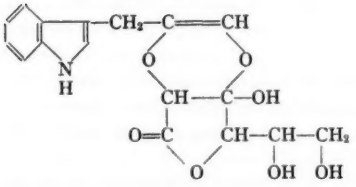
action. Such secondary effects, for example on numerous enzyme actions, are of necessity carefully integrated and highly organised to produce growth and differentiation. Since enzymes are inevitably involved one way or another in growth, they will be involved in the action of a hormone on a cell. Similarly, essential metabolites, e.g., ATP or water are inevitably involved. From a consideration of these points, Hechter (80) suggests that the questions which have guided experimental work for the past decade or so are the "wrong" questions in the sense that they cannot give answers leading to a knowledge of the mechanism of hormone action. The real question should be whether the primary action of the hormone, probably controlling one step in a chain of reactions, need necessarily involve an enzyme, and to this question the answer need not be "yes." The difficulty here is that the alternative hypothesis, involving presumably a "hormone signal" or excitation effect, is not readily susceptible to experimental attack with present techniques. Nevertheless, such an effect could easily be a master reaction controlling protoplasmic activity. A similar suggestion is that hormones reorientate the cytoskeleton, causing modification of several enzymatic reactions simultaneously (161). If this happens, it follows that search for some one enzymic reaction which a hormone modifies is doomed to failure.

These concepts of hormone action will probably become open to investigation as our understanding of the nature of cellular membranes, energy transfer mechanisms, and organization of cell contents increases.

Since this article was prepared for publication, important work done in Czechoslovakia on a substance called ascorbigen has come to the author's notice (169a, 169b). This is a most interesting compound which liberates ascorbic acid on acid hydrolysis, while on alkaline hydrolysis it gives rise to IAA. Work on the chemical structure suggests either formulae I or II (Fig. 1) for ascorbigen.



I.



II.

FIG. 1

A range of higher plants has been examined, but it has so far been located only in the Cruciferae, especially in the Brassicas. It is readily soluble in water and ethanol but less so in ether and chloroform.

Such a compound is of great interest in connection with the unknown auxin complexes reviewed here. It could explain some results listed in Table III, and discussed in the text in the section on unidentified auxins. Furthermore, the close interrelationship which has been demonstrated between auxin metabolism and ascorbic acid metabolism (p. 70) is explicable in terms of this compound. Obviously, it is necessary to determine the distribution of ascorbigen in plant tissues, and its possible physiological effect in plants.

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DESTRUCTION OF AUXIN[1,2]

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A considerable amount of attention has been given to both the biochemistry and physiology of auxin destruction in plants since the review of this topic by Gordon (54) in 1954; the present review deals largely with the progress in this field since Gordon's review. The greatest amount of recent work has concerned the oxygen-consuming enzymes which destroy indole-3-acetic acid (IAA), and most of the discussion will necessarily be devoted to this aspect of the subject. An effort will also be made to survey other destruction mechanisms which are known, both for IAA and for other auxins. Finally, the relation between these processes and the physiology of plants will be considered.

Chemistry of IAA Destruction

Photooxidation.—Brauner (17) followed riboflavin-sensitized photooxidation of IAA simultaneously with (*a*) a *Helianthus* curvature test, (*b*) the Salkowski color reaction, (*c*) the change in pH, which accompanies decarboxylation, and (*d*) the Hopkins-Cole color reaction for the indole nucleus. He found that the changes in different assays did not run parallel, and interpreted the results as indicating that IAA photodestruction was occurring in three steps, the first converting IAA to a physiologically inactive, Salkowski-positive acid, the second involving decarboxylation and loss of Salkowski reactivity, and the third forming a product of weak Hopkins-Cole reactivity. This conclusion might be partly questioned, because the bioassay showed evidence of being affected by riboflavin, and by illumination of the solutions, in such a way as to make calibration with pure IAA unreliable. However, other evidence also indicates the existence of a sequence of reactions in photooxidation.

Fischer (40) reported the detection, by chromatography and electrophoresis, of indole-3-carboxaldehyde (hereafter called indolealdehyde) as a production of both ultraviolet and of riboflavin-sensitized photooxidation of IAA. From products of the latter reaction it was isolated by chromatography and identified chemically. Another substance "E," having an R_F between that of IAA and that of indolealdehyde, was also detected with the nitrite reagent (similar to the Salkowski reaction); it was isolated and reported to be identical with indole-3-glycolic acid. Fischer (40) ac-

[1] The survey of literature pertaining to this review was concluded in October, 1957.

[2] The following abbreviations will be used: DPNH, diphosphopyridine nucleotide (reduced form); IAA, indole-3-acetic acid; IAN, indole-3-acetonitrile; 2,4-D, 2,4-dichlorophenoxyacetic acid.

cordingly formulated the photooxidation of IAA as

$$I\cdot CH_2\cdot COOH \rightarrow I\cdot CHOH\cdot COOH \rightarrow I\cdot CO\cdot COOH \rightarrow I\cdot CHO \qquad 1.$$

(where the indole nucleus is represented by I), a reaction sequence which had been suggested for the enzymatic oxidation by Goldacre (49). Fischer (40) did not report the yield of indolealdehyde, so one could not know whether it was in fact the main reaction product, as required by reaction 1. Furthermore, Table 2 of his paper gives the apparently misleading impression that indoleglyoxylic acid was detected in products of photooxidation; no such statement appears in the body of the paper, and since the R_F and electrophoretic mobility of this substance and IAA are stated to be identical, it is certainly questionable whether indoleglyoxylic acid could have been detected. Some evidence was presented (also in 13) that indole and skatole may also be formed in small amounts. Two neutral, colored materials were isolated after prolonged irradiation and thought to be polymeric pigments derived from the condensation of indolealdehyde, skatole, and indole. The analyses reported for these (7.27 and 5.74 per cent N, respectively) rather speak against this idea, because the N content of such condensates would be expected to be greater than 10 per cent; actually, the reported C/N ratio indicates that these substances may have been derived in part from degradation products which had lost nitrogen. In the products of ultraviolet photodestruction of IAA in ethanol, Mayr (94) detected chromatographically substance "E," which was found to have no activity in the pea curvature test, but among the products in the neutral R_F region, biological activity equivalent to about 2 per cent of the initial IAA was found, when 90 per cent of the IAA had been destroyed. It seems possible that either the formation of the ethyl ester of IAA (the solvent was EtOH), or even of indoleacetamide (by transfer of N from a degraded molecule), might explain this observation.

A question surrounding scheme 1 concerns the identification of indoleglycolic acid, for this substance, according to several workers (14, 57, 113), runs at an R_F lower than IAA in the type of solvent used by Fischer (40), while "E" runs above IAA. Unfortunately Fischer did not report the method of preparation of the synthetic material (stated to be indoleglycolic acid) with which "E" was compared and found to be identical. The true identity of "E" is as yet uncertain.

Spectrophotometric study of the ultraviolet photooxidation reaction (113) has provided evidence of the accumulation and subsequent destruction of an intermediate which may be the same as Fischer's "E," and of the formation of indolealdehyde as a final product. However, not more than about 25 per cent yield of indolealdehyde was obtained, apparently because photodestruction of the intermediate yields a mixture of products, of which indolealdehyde is only one. The yield of indolealdehyde was even less at irradiation wavelengths above 280 mμ, and above 300 mμ a product very similar to that of enzymatic oxidation of IAA (115) was formed. This

effect could be attributed to the existence of a second intermediate on the path leading to indolealdehyde, this intermediate being unstable, and, although capable of being converted in part to indolealdehyde by irradiation, decomposing also by a competing dark reaction into the products characteristic of the enzyme reaction. Whether this intermediate is the same as the one observed in the enzyme reaction (111) is not yet certain. But since Stutz (133, 135) had already found that the enzymatic reaction could be diverted to formation of indolealdehyde (cf. below), it seems likely that the two processes have at least one intermediate in common. Evidently it is impossible to formulate the photooxidation reaction as a stoichiometric conversion of IAA into indolealdehyde, as in reaction 1.

Contributions to the mechanism of IAA photooxidation since the work of Brauner (17, 18), reviewed by Gordon (54), are not numerous. Goldacre (50) studied photooxidation of IAA by dialysable constituents of pea homogenates, finding that several compounds active as sensitizers were present in etiolated peas, while in light-grown peas inhibitors of photooxidation tended to mask the activity of any sensitizers present; only a minor proportion of the photodestructive activity could be attributed to riboflavin. He studied the kinetics of the process, which are complicated by inactivation of the sensitizer, and also examined the effect of pH on the reaction, as did Shugar (123). Mer (95) found that riboflavin nucleotides would sensitize photodestruction, and Andreae (5) found that scopoletin, also a natural constituent of plants, would likewise. He studied the photooxidation of Mn^{+2}, which was sensitized by riboflavin, but required a phenol or, interestingly enough, IAA, in addition. Although the capacity of riboflavin to sensitize photooxidation of IAA has been repeatedly confirmed, it was recently concluded by Waygood & Maclachlan (147) that "riboflavin neither activates nor sensitizes IAA oxidation, but on the contrary protects the hormone in darkness and inhibits its breakdown." Apparently this was meant to refer only to the special effects of riboflavin and light on enzymatic oxidation, since it had been found that riboflavin light-reversibly inhibits the enzymatic oxidation. Actually, the light intensities and riboflavin concentrations employed in the enzyme experiment reported here were such as might be expected to cause rapid photodestruction of IAA (17), yet no mention was made of a control with riboflavin and IAA alone in light. Undoubtedly such a control was run, but the published data do not allow one to judge with confidence how much of the effect might have been due to direct photodestruction.

Gordon & Weber (55) examined the kinetics of IAA destruction by x-rays, and showed that they are consistent with reaction of both IAA and its destruction products with radicals generated in the solvent by the radiation; the products thus have a protective effect on the remaining IAA. Curry, Thimann & Ray (27) determined the action spectrum for photoinactivation of IAA by ultraviolet light, and found it to resemble closely the absorption spectrum of IAA, as would be expected.

Enzymatic oxidation products.—Formation of indolealdehyde previously appeared to be the most likely pathway for enzymatic oxidation of IAA, a process which has approximately the same stoichiometry as the photooxidation ($IAA + O_2 \rightarrow CO_2 +$ Product). Manning & Galston (92) found that at least two substances, giving pink colors with $FeCl_3$, were formed from IAA in pea homogenates, neither of them being indolealdehyde; nor did the latter give rise to the observed products if added to the enzyme preparation. These authors suggested that IAA could be oxidized in a manner analogous to the well-known pathway of tryptophan metabolism in animals (82), in which the indole ring is opened between the 2- and 3-carbons by oxidation to give formylkynurenine. This enzymatic reaction has the same oxygen stoichiometry as IAA oxidation; the analogous product from IAA, after decarboxylation, would be *o*-formylaminoacetophenone. Manning & Galston (92) found, however, that neither of the above products of IAA oxidation was identical with this compound, and suggested that at least one of the products was a hydroxylated aminoacetophenone. This interesting hypothesis will be considered further below; but it now seems unlikely that the particular product they were dealing with had this structure, because the ultraviolet spectra observed showed no indication of the characteristic maxima of acetophenone derivatives. Stowe, Ray & Thimann (130) found that ultraviolet and infrared spectra of the product formed from IAA by the *Omphalia* enzyme were rather similar to those of the oxindole nucleus, and suggested that the product might be 3-methyldioxindole. However, this compound never could be isolated from the product (115), and it was found that, as with the pea homogenate (92), several neutral substances were actually formed, two of which gave a pink Salkowski (or $FeCl_3$) color, and may retain the indole nucleus. Indolealdehyde, however, did not appear to be formed in appreciable amounts. When IAA labelled with C^{14} in the 2-carbon of the indole ring was used as substrate, the label was entirely retained in the products. The products appeared to be unstable during manipulation, and analyses indicated that further oxidation may have been occurring. The formation of several products does not appear to be attributable to the action of several enzymes, because several lines of evidence indicated that only one enzyme was present (111, 112).

Stutz (134) found that at least five products were formed from IAA by a highly purified enzyme obtained from *Lupinus* seedlings. Here also, two Salkowski-positive neutral substances were detected, and in addition, an apparently acidic Salkowski-positive substance whose R_F suggests it may be the same compound as Fischer's "E" of photooxidation, discussed above. Using C^{14}-labelled IAA, Stutz (135) found that whereas the methylene carbon of the sidechain was retained in the products, the carboxyl group was substantially lost as CO_2, except in the case of another acidic, but Salkowski-negative, product (the Salkowski-positive acid similar to "E" may not have been studied in this respect). As with the *Omphalia* enzyme products, some of the materials formed could not be detected except by

using labelled substrate, since they gave no color reactions or fluorescence. Also, there was indication that the products were unstable to preparative procedures or chromatography.

The intermediate.—It can be seen that there is considerable general similarity between the products formed from IAA by the several enzymes recently investigated, and that more than one substance was formed in each case. A possible explanation for this lies in the finding (111, 114) that in the case of the *Omphalia* enzyme and of horseradish peroxidase, the changes in ultraviolet spectrum which accompany the conversion of IAA into the product indicate the formation and disappearance of an intermediate. The enzyme was found to catalyze only the conversion of IAA into the intermediate, which, being an unstable substance, decomposes spontaneously at a rather rapid rate to give, it appears, at least two and probably more final products. Since this takes place in dilute solution, it is not surprising that the products are reactive, and undergo further changes when concentrated and manipulated. Since the intermediate is the actual product of the enzymatic reaction, its identity may really be of greater interest than that of the mixture of final products. All that is known so far is that the intermediate is neutral and has the indole (not oxindole) structure, is at the same oxidation state as the final products, and is not indolealdehyde. Despite the complex changes in spectrum which accompany IAA oxidation, a direct spectrophotometric method for following the reaction has been worked out (111).

Enzymatic formation of indolealdehyde.—The *Omphalia* enzyme oxidized indole-3-isobutyric acid more rapidly than IAA, and with similar stoichiometry (115), so there appeared to be no possibility that IAA was oxidized stepwise in the sidechain (as in equation 1), nor that indolealdehyde could be formed, because indoleisobutyric acid has the same structure as IAA except that the two hydrogens of the sidechain methylene carbon are replaced by methyl groups, blocking any oxidation. However, Racusen (110) detected small amounts of a 2,4-dinitrophenylhydrazine-positive substance, which he believed was indolealdehyde, in the products of IAA destruction by the *Lupinus* enzyme. Recently Stutz (133, 135) discovered that if cytochrome-*c* and a cytochrome oxidase-containing homogenate of rat liver is added to the lupine enzyme, amounts of indolealdehyde corresponding to as much as 50 per cent yield from IAA are formed. This oxidation was not catalyzed by the homogenate plus cytochrome-*c* alone. Furthermore, indoleglycolic acid was oxidized rapidly by the lupine enzyme and converted almost quantitatively into indolealdehyde, at an oxygen stoichiometry approximating $\frac{1}{2}$ O_2 per mole aldehyde formed. Stutz pointed out that if the action of the cytochrome system were to cause indoleglycolic acid to be formed from IAA by the lupine enzyme, indolealdehyde would be formed automatically as product. The mechanism by which the added cytochrome system brings about this effect is not at all clear; the cyto-

chrome oxidase-containing preparation being a simple homogenate, it could be that constituents other than cytochrome oxidase are responsible, for example, substances capable of reducing cytochrome-*c* which might then reduce an IAA oxidation intermediate to indoleglycolic acid. It was not actually established that there was hydrogen transfer to oxygen via the cytochrome system; but the requirement for cytochrome-*c* is suggestive of this possibility, and its further exploration should be highly interesting. Cytochrome-*c* actually inhibited the oxidation of IAA, and the highest yields of indolealdehyde (based on the IAA destroyed and oxygen consumed) were only obtained under severe inhibition of the over-all rate, as if the intermediate diverted to indoleglycolic acid (or indolealdehyde) formation served normally to propagate the oxidative reaction. As to the possible relationship of these findings to the pathway of IAA oxidation in intact plants, it may be mentioned that Fischer (40) reported detecting indolealdehyde in several plant extracts; however, Good, Andreae & Van Ysselstein (53) did not detect indolealdehyde among the products of IAA metabolism in various plant stem sections, and an acidic product of R_F somewhat below IAA appears, from its reported color reactions, not to have been indoleglycolic acid (cf. 57).

Stutz (135) also found that indoleisobutyric acid was rapidly oxidized by the lupine enzyme, but would not give substantial yields of indolealdehyde even if the cytochrome system were added. It may be noted that the 2,4-dinitrophenylhydrazone assay values found for the products of indoleisobutyric acid oxidation are very close to those found for oxidation of IAA with the lupine enzyme alone, which suggests that the latter values do not necessarily indicate the formation even of trace amounts of indolealdehyde from IAA when the cytochrome system is not added.

Primary oxidation.—Stutz (135) suggested that these various results could best be interpreted as indicating primary oxidation of the indole nucleus, followed by oxidation of the side chain by the nucleus, depending on the susceptibility of the methylene C to oxidation and on the influence of added hydrogen carriers such as the cytochrome system. Stutz found that 1-methylindole-3-acetic acid (nitrogen atom blocked) was rapidly oxidized by the lupine enzyme, whereas 2-carboxyindole-3-acetic acid was oxidized by the crude homogenate, but not by the purified enzyme; whether decarboxylation to IAA, freeing the 2-carbon, was involved is not known.

An alternative suggestion concerning the site of primary oxidation was made by Maclachlan & Waygood (91) for oxidation of IAA by Mn^{+3} to give a product which these authors considered to be the same as is formed in enzymatic oxidation; as shown in equations 4 and 5 (p. 94), they envisioned primary oxidation at the carboxyl group, followed by decarboxylation to give a skatolyl radical. It is evident that this mechanism could explain the rapid oxidation of indoleisobutyric acid even though there is no α-hydrogen atom, and also the generally encountered fact that only carboxylic acids are oxidized, even the ethyl ester of IAA being inert (115). However,

in the case of indole-3-propionic acid, the oxidation of which (by horseradish peroxidase) was found by Kenten (74) to have properties similar to those which are considered evidence for the sequence of reactions beginning with equation 4, no CO_2 was formed. Equation 4 thus does not appear to represent a general mechanism for enzymatic oxidation of indole-3-alkylcarboxylic acids, and it would seem that if there is one primary site of oxidation among the compounds studied, it must lie in the indole nucleus, as Kenten (74) also suggested. The requirement for a free carboxyl group could be explained as being involved in the attachment of the substrate to the enzyme. That indoleglyoxylic acid and indole-3-carboxylic acid are not oxidized (135) is not really surprising, because the electronic structure of their indole nuclei, as evidenced by ultraviolet spectra, is quite different from that of IAA or indole itself.

Other oxidations.—Manning & Galston (92) suggested that IAA could be destroyed by hydroxylation followed by oxidation of the indole ring to the acetophenone structure. While the products formed in their enzyme preparation may not have had this structure (see above), both hydroxylation and ring opening are possible routes of IAA destruction which may warrant further exploration. The 5-hydroxyindole alkaloid bufotenine has been isolated from at least two plant sources (132, 155), indicating that a mechanism for hydroxylation of the indole nucleus must exist in plants, as it does in animals (141). It was found by Dalgleish (28, 29) that IAA can be hydroxylated to its 5- and 7-hydroxy derivatives by the nonenzymatic Fe^{+2}-ascorbic acid hydroxylating system. Since these hydroxyindoleacetic acids are known to show only weak auxin activity (32), such a reaction could certainly be considered auxin inactivation. Mason *et al.* (93) reported that hydroxylation of aromatic compounds can be made to occur enzymatically in the oxidation of dihydroxyfumarate by peroxidase. Enzymatic hydroxylation of IAA may, therefore, be anticipated.

The possibility that the acetophenone structure may be formed from IAA should also not be ignored. It is reasonable to suggest that the IAA-oxidizing reactions so far studied may be compared with the oxidation of tryptophan by lactoperoxidase or verdoperoxidase, which has the same oxygen stoichiometry, and yields as yet unidentified, in part colored and probably polymeric, products (81). Tryptophan peroxidase, on the other hand, accomplishes the same degree of oxidation with the formation of the acetophenone derivative formylkynurenine. Wiltshire (156) obtained spectroscopic evidence of the formation of the kynurenine structure from tryptophan in crude pea homogenates, but observed only the unidentified degradation products when the purified tryptophan-oxidizing peroxidase was used. He thought that a relatively labile enzyme might be needed for formation of the kynurenine structure.

Houff *et al.* (66) proposed that the red oxidation product of IAA formed in the Salkowski color reaction is N-hydroxyindoleacetic acid. The evidence does not appear to establish this particular structure with any

certainty, and seems to be largely that N-substituted indole derivatives did not give the color reaction, and that two equivalents of Fe^{+3} were eventually reduced. Fearon & Boggust (39) stated that four oxidizing equivalents were required for maximum color development in an apparently closely similar oxidation of IAA, and they proposed an entirely different structure for the colored product (a structure which is also excluded by N-substitution). On the other hand, the originally characterized N-hydroxyindole-2-carboxylic acid appears to have been a colorless or near colorless compound (118).

Maclachlan & Waygood (90) reported that oxidation of IAA by Mn^{+3} in acid solution gives a purple precipitate; this may be the same material as was prepared by Houff *et al.* (66). Nitrous acid also oxidizes IAA to a red color (39, 40) which is very similar to that of the usual Salkowski reaction; this is interesting because, like Mn^{+3}, nitrite has been found (139) to initiate an oxygen-consuming oxidation of IAA at near-neutral pH which resembles enzymatic oxidation in several respects. Thus, there may be some chemical connection between the Salkowski reaction and the enzymatic oxidation. This can be extended to the observations of Platt & Thimann (108) that some of the polyphenolic compounds which inhibit enzymatic oxidation also inhibit the Salkowski reaction, and H_2O_2 and light can accelerate it. Kinetic properties of the Salkowski reaction have also been studied by Pilet (101).

Conjugation.—An entirely new aspect of auxin metabolism has been opened up by the discovery of Andreae *et al.* (7, 53) that in plant tissues IAA may become conjugated with aspartic acid to give the peptide indoleacetyl-N′-aspartic acid. This work is being reviewed by Bentley in this volume. Since the conjugate was found to have extremely weak auxin activity in the pea test (7), its formation in peas must not be readily reversible, and constitutes a type of auxin inactivation or, as the authors suggest, detoxification. However, the conjugate was also formed in oats (53), on which it was found to have auxin activity comparable with that of IAA (7), so its formation there could not be considered auxin inactivation. But with oats and other grasses, indoleacetamide, a derivative with weak auxin activity, was also formed in considerable quantity from IAA. Enzymes which form these substances have not yet been obtained from plants, but they undoubtedly exist and should form interesting objects for study. The conjugates do not seem yet to have been detected as natural components of plants.

Enzymatic Mechanisms of IAA Destruction

Flavoprotein-peroxidase hypothesis.—All of the enzymatic IAA-destroying reactions which have been investigated in this respect have showed a requirement for oxygen, and a stoichiometric uptake of one mole O_2 per mole IAA destroyed. The IAA-destroying enzymes in plant tissue homogenates are, therefore, often called "IAA oxidases." With one exception

which is mentioned under the final heading of this section, the enzyme preparations which have been characterized have also been found to show typical peroxidase activity, that is, to catalyze the oxidation of phenols and certain other substrates using H_2O_2 as electron acceptor. A hypothesis which has figured prominently in the interpretation of IAA oxidation in plant homogenates is that the "oxidase" is a coupled system consisting of a peroxidase using H_2O_2 as oxidant, and a flavin enzyme forming H_2O_2 from oxygen. This was proposed by Galston, Bonner & Baker (47). It resembles the interpretation of the enzymatic oxidation of tryptophan to formylkynurenine suggested by Knox and Mehler (82), and can be formulated as follows:

$$\text{IAA} + H_2O_2 \xrightarrow{\text{peroxidase}} P_1 + 2H_2O \qquad 2.$$

$$P_1 + O_2 \xrightarrow{\text{flavoprotein}} P_2 + H_2O_2 \qquad 3.$$

This serves to explain the oxygen stoichiometry as well as a variety of evidence that H_2O_2 is required, such as the inhibitory effect of catalase and polyphenols, and the stimulation which can be obtained with added H_2O_2 under certain conditions (47, 49). It would also seem capable of accounting for the dependence of the reaction rate on oxygen pressure over a wide range, as found by Wagenknecht & Burris (142), for this is a property typical of most flavin enzymes. The principal direct evidence presented by Galston *et al.* (47) for the participation of two separate enzymes, was a procedure involving precipitation of protein at pH 2.5 which, when redissolved, had low activity in IAA oxidation alone but showed substantial IAA-oxidizing activity when combined with the supernatant. The precipitation followed Warburg & Christian's (144) method for splitting D-amino acid oxidase into apoenzyme (precipitate) and flavin-adenine dinucleotide (supernatant); evidence was obtained (47) of dialyzable flavin in the supernatant, which also contained the peroxidase. It would, of course, have been only a fortunate coincidence if reversible splitting and precipitation of a different flavin enzyme with unknown prosthetic group could be obtained under the same conditions as with D-amino acid oxidase; it seems possible that the results could have been due to some other cause, such as separation of an H_2O_2-producing enzyme from its substrate, or involving the phenolic cofactors (see below). Had it been possible to reconstitute activity from the precipitate, a known flavin, and a peroxidase further purified from the supernatant, we would have less room for doubt that a flavoenzyme involved in the main reaction had been detected. Unfortunately, this experiment does not seem to have been pursued further with "IAA oxidase" preparations in subsequent years, except by Kenten (73), who repeated the precipitation on a purified horseradish peroxidase preparation and found little diminution of activity.

Galston *et al.* (47) realized that the IAA-oxidizing reaction resembles

in a number of respects the oxygen-consuming oxidation of dioxymaleic acid by purified peroxidase, which had been known for some years (136). This oxidation has been formulated (25) in terms somewhat similar to equations 2 and 3, except that oxidation of dioxymaleic acid itself is supposed to occur by both "peroxidase" and "oxidase" reactions, and both of these are supposed to be catalyzed only by peroxidase. Galston *et al.* (47) did find that peroxidase preparations alone would oxidize IAA. Subsequent work has concentrated on the oxidation of IAA by peroxidases alone, and to what extent (if at all) a flavoprotein-peroxidase coupled system contributes to IAA oxidation in homogenates remains unknown. It does seem that most of the properties of IAA oxidation in homogenates can at present be accounted for by the behavior of peroxidases alone. In the case of tryptophan oxidation, the flavoenzyme-peroxidase scheme was given up entirely, since fractionation experiments never were successful; the reaction is considered to be catalyzed by the single enzyme "tryptophan peroxidase"(81).

Peroxidases and H_2O_2.—Kenten (73) found that the oxygen-consuming oxidation of IAA by purified horseradish peroxidase was stimulated enormously by low concentrations of the same monophenols which had been found by Goldacre, Galston & Weintraub (52) to stimulate IAA oxidation in pea homogenates. Similar effects were observed with IAA oxidation by homogenates of bean roots (73). The bean roots contained a dialyzable (ultrafiltrable) promotive factor which was shown to resemble the phenolic activators in that activity could be destroyed by prior treatment with peroxidase plus H_2O_2, an indication that the cofactors are substrates of peroxidase. Monoamines and resorcinol also promoted, but *o*- and *p*-diphenols, polyphenols, and diamines were strongly inhibitory; this could be reversed by adding a small amount of H_2O_2. Since the polyphenols are known to be substrates of peroxidase, it seemed likely that the addition of H_2O_2 had caused rapid oxidation of the inhibitors by peroxidase, and that the inhibition of IAA oxidation caused by them could be attributed to their tendency to remove H_2O_2 from the system by being rapidly oxidized "peroxidatively." Inhibitions were also obtained by adding catalase; Kenten (73) felt that the evidence favored formation of and dependence on H_2O_2 in the IAA-oxidizing reaction; i.e., something like equations 2 and 3 but with peroxidase catalyzing both steps; this view was in harmony with previous interpretations of the effects of catalase and polyphenols (6, 47, 49).

As far as polyphenols are concerned, however, it should be noted that polyphenol inhibitions of photooxidation (18) and chemical oxidation (108) of IAA are known, in neither of which cases does the participation of peroxidase come into question, and in fact inhibition by polyphenols is characteristic of oxidation processes involving free radicals (44). Thus, it cannot be unequivocally concluded that inhibition by polyphenols involves direct interaction with peroxidase and H_2O_2 (even though reversal of inhibition by H_2O_2 may). This applies even when kinetics which approximate Lineweaver-Burk "competitive" inhibition have been observed (e.g., 3,

49, 80), because the kinetics of possible interactions involving radicals could also be "competitive" with IAA, which certainly reacts with radicals in photo- and x-ray oxidation (55). A similar difficulty is encountered with the suggestion of Waygood *et al.* (150) that inhibition by polyphenols is due specifically to their capacity to reduce Mn^{+3}; these authors actually presume the involvement of radicals in the oxidation, and such radicals would certainly react with polyphenols. The observation least suspect of this ambiguity would appear to be that of Platt (107), who studied destruction of IAA at *Avena*-test concentration (6×10^{-7} *M*) by horseradish peroxidase, where it appeared to be stoichiometric in added H_2O_2, one mole of IAA being destroyed per mole of H_2O_2 added. When the peroxidase was added to solutions 6×10^{-7} *M* in IAA, H_2O_2 and any of various polyphenols, residual amounts of IAA were found which could be taken to indicate that these phenols react with peroxidase·H_2O_2 about as rapidly as IAA does. Unfortunately, the data do not actually show that the reactions had gone to completion (an assumption equally critical for the stoichiometric interpretation of the reaction), although this may have been established separately. Platt stated that the same enzyme preparations were found to destroy IAA in the absence of added H_2O_2 if the IAA concentration were greater than about 10^{-5} *M*, and he evidently believed that the mechanism was different; how an increase in concentration of one order of magnitude should alter the mechanism is a problem. It will be noticed that the reported H_2O_2 stoichiometry is equivalent to one-half the oxygen stoichiometry observed by all other workers, and would correspond to the operation of reaction 2 alone. These interesting observations appear to deserve being pursued further, for example to discover whether oxygen was in fact not involved. The occurrence of a peroxidase-catalyzed oxidation of IAA by H_2O_2 independent of oxygen, i.e., reaction 2, actually has never been demonstrated in any system, and seems only to have been looked for once (112); it is critical to the idea of cyclical H_2O_2 utilization (equations 2 and 3).

Siegel & Galston (127) reported promotive effects of Mn^{+2} and of 2,4-dichlorophenol on the oxidation of pyrogallol by sections of pea stems and roots, which were interpreted as due to increase in the formation of "peroxides" in the tissues; they felt that such an effect might explain the promotive effect of these substances on IAA oxidation. The effects were not reported for homogenates, where, of course, they might be expected if they account for effects on the rate of IAA destruction in homogenates. It was also found that IAA added to stem sections promoted pyrogallol oxidation, which the authors felt demonstrated the formation of H_2O_2 from IAA and supported the idea that "IAA oxidase" is an oxidase-peroxidase system coupled through H_2O_2. However, at the concentration of pyrogallol used (0.01*M*), IAA oxidation in homogenates would have been totally inhibited, making it difficult to see how this observation should be related to the evidence for the mechanism in homogenates. Moreover, if the absolute amount of "H_2O_2" formed is reckoned on the same basis as that for some flavin

enzyme substrates which were supplied in a different experiment, it appears that with the lowest amount of IAA used, an amount of H_2O_2 equivalent to well over half the IAA added had been formed when the experiment was ended (even if the use of the figure 2 instead of 3 for the H_2O_2/purpurogallin stoichiometry in the original calculations is neglected). This must mean that even had all the IAA been oxidized, at least half of it would have given rise to an excess equivalent of H_2O_2. Had all the IAA been oxidized in a coupled system like equations 2 to 3, no increase in H_2O_2 would have resulted, since the system would use up as much H_2O_2 as it regenerated. This experiment must then represent either the detection of an IAA-destroying reaction which produces H_2O_2 and which, unlike the supposition for homogenates, neither uses it nor is inhibited by polyphenols; or it is due to secondary effects of IAA on the tissues rather than to IAA destruction. It is difficult to decide which, because it is not known how much of the IAA did actually disappear in the experiment, nor with what degree of certainty the observed pyrogallol oxidation must be considered to be caused by H_2O_2.

Manganese effects.—Kenten (73) found that added Mn^{+2} (10^{-5} to 10^{-3} *M*) increased the rate of IAA oxidation by horseradish peroxidase some two- to threefold. However, at the higher Mn concentrations the reaction was inhibited initially, the rate gradually increasing and finally exceeding that of the control. With the latter, the rate declined with time and the reaction failed to reach completion unless relatively small amounts of IAA were used; this may have been due to inactivation of the enzyme, since addition of further enzyme was found to increase the total oxygen uptake. At the higher concentrations of added Mn, this decline in rate was much less rapid, the enzyme apparently being protected against inactivation; similar effects can be seen with the lupine (134) and *Omphalia* (115) enzymes. Kenten did not associate the action of Mn definitely with that of the stimulatory phenols, but he did find that the more purified preparations of horseradish peroxidase showed less stimulation by Mn. Hillman & Galston (63) found that with a much diluted pea homogenate, inhibition by added Mn^{+2} was observed at 10^{-6} *M* 2,4-dichlorophenol, while promotion, apparently as much as twofold, was found at 10^{-5} *M* or greater phenol. They considered that the endogenous concentration of phenols would thus determine whether stimulation or inhibition by Mn^{+2} were observed with enzyme preparations to which phenols were not added. A rather similar observation was made by Pilet (99) with *Lens* root homogenates. In Kenten's (73) experiments with horseradish peroxidase, positive effects were obtained with 10^{-5} *M* phenols, so it seems unlikely that endogenous phenols, if present at all, could have been this concentrated, yet, as mentioned, promotions by Mn were obtained. However, Kenten's results are not actually in conflict with those of Hillman & Galston (63), when it is considered that the latter workers were dealing with a total amount of IAA destruction equivalent, in Kenten's experiments, to the initial period during which added Mn strongly inhibited. Increase in

concentration of phenol, then, may overcome the initial inhibitory effect of Mn; but whether it alters the ultimate rate effects of Mn, or only makes these appear earlier, cannot be decided from these experiments, although the former seems more than likely.

Reinert, Schraudolf & Reinert (116) have discovered that another effect of Mn can be to shift the pH optimum of IAA destruction in dialyzed pea homogenate from about pH 5 (no Mn) to about pH 4 (1.7×10^{-3} *M* Mn). The resulting pH curve rather resembles that reported by Gortner & Kent (56) for IAA oxidation in pineapple homogenates, where 5×10^{-3} *M* Mn was used. The shift in form of the pH curve with the pea homogenate (116) was due to much stronger promotion by Mn at acidic than at near-neutral pH. Both the form of the pH curve and the strong effect of Mn (tested at acidic pH) have been thought to distinguish the pineapple enzyme as a type different from the IAA-oxidizing enzyme of peas; since when the pea homogenate was tested under comparable conditions, it gave essentially similar effects, Reinert *et al.* feel that the supposed distinction is without foundation. They presented evidence that the effect of chelating agents, also thought to distinguish the pineapple enzyme, may depend upon the conditions used. It would be well, of course, to confirm that in the absence of added Mn, pineapple homogenate still does not show an optimum at pH 3.5 to 4, before this matter is considered settled, because in the case of the *Omphalia* enzyme (115) an optimum at pH 3.5 was found in the absence of added Mn, and indeed was found to be characteristic of the peroxidase responsible for the IAA-oxidizing reaction (112). And on the other hand, Maclachlan & Waygood (91) observed an optimum at pH 5 with a wheat leaf homogenate, using 10^{-3} *M* Mn^{+2} in the presence of various phenolic cofactors, but an optimum closer to pH 4 when an extract of the dialyzable fraction of the homogenate was used as cofactor. The form of the pH curve with Mn^{+2} may, thus, also depend on the cofactor or its concentration, or both. These interactions appear to present a multidimensional problem. Whether or not they are involved in the interesting finding of Reinert *et al.* (116) that dialyzed pea homogenate without Mn shows a subsidiary optimum at pH 4, will no doubt be clarified by further work.

Oxidation of manganese.—Kenten (73) noticed that those phenols which stimulated the oxidation of IAA by horseradish peroxidase were the same ones as he and Mann (76) had found to be required in the peroxidase-catalyzed oxidation of Mn^{+2} to Mn^{+3} by H_2O_2, and to promote the oxidation of certain dicarboxylic acids, including dioxymaleic, by peroxidase plus Mn^{+2} (77). The oxidation of Mn^{+2} was interpreted as an oxidation of the phenol by peroxidase·H_2O_2, followed by reduction of the phenol oxidation product by Mn^{+2} to give Mn^{+3} and the phenol. Kenten & Mann (77) suggested that oxidation of Mn must be involved in the peroxidase-catalyzed oxidation of dicarboxylic acids mentioned above. Although Kenten (73) considered that oxidation-reduction of the phenols was most likely involved in their promotive effects on IAA oxidation, he did not suggest that oxida-

tion of Mn was involved, perhaps because added Mn was not required. But he considered this possibility in connection with the oxidation of indolepropionic and indolebutyric acids by the same enzyme preparation (74), for which added Mn was found to be essential. The oxidation was, however, only weakly stimulated by phenols. Inhibitions were obtained by adding catalase. In the presence of indolepropionic acid, peroxidase without Mn caused a doubling of the oxygen uptake rate of a glucose oxidase plus catalase system, as if it were competing with catalase for the H_2O_2 formed by the oxidase, and carrying out a reaction analogous to equation 2. It was suggested, therefore, that added Mn may make possible the oxygen-consuming oxidation by promoting the formation of H_2O_2.

Waygood, Oaks & Maclachlan (149, 150) observed promotive effects of Mn and phenols on IAA oxidation by wheat leaf homogenates, resembling many of the effects already described. They discovered (90) that Mn^{+3} (ethylenediamine-tetraacetic acid complex) caused destruction of IAA accompanied by CO_2 production and O_2 uptake, giving a product which they believe is the same as that formed by the enzymatic oxidation (its properties have not yet been reported). They suggested (150) that the various effects of phenols and added Mn could be explained by supposing that enzymatic oxidation of IAA does not involve interaction between IAA and the enzyme at all, but rather the oxidation of IAA by Mn^{+3}, forming Mn^{+2} and a radical which reacts with oxygen, the oxidized product serving as oxidant for the oxidation of the phenol by peroxidase, and thereby the reoxidation of Mn^{+2} by the phenol, as in the Kenten-Mann system mentioned above. This ingenious idea has been formulated explicitly as follows (91):

$$I\cdot CH_2COOH + Mn^{+3} \longrightarrow I\cdot CH_2COO\cdot + Mn^{+2} \quad 4.$$

$$I\cdot CH_2COO\cdot \longrightarrow I\cdot CH_2\cdot + CO_2 \quad 5.$$

$$I\cdot CH_2\cdot + O_2 \longrightarrow I\cdot CH_2OO\cdot \quad 6.$$

$$I\cdot CH_2OO\cdot + ROH \xrightarrow{\text{peroxidase}} I\cdot CH_2O_2H + RO\cdot \quad 7.$$

$$RO\cdot + Mn^{+2} \longrightarrow ROH + Mn^{+3} \quad 8.$$

Here I represents, as before, the indole nucleus, and R the nucleus of the monophenol. Reaction 6 is founded on the well-known interpretation of free-radical chains in organic auto-oxidation processes, and it attractively explains the mole-for-mole oxygen uptake in IAA oxidation. Equation 8 is based on Kenten & Mann's (75) interpretation of Mn^{+2} oxidation, with the added refinement that the phenol oxidation product is indicated as an aryl radical, the only monophenol oxidation product capable of reversible oxidation-reduction. The specific formulation of the remaining reactions is hypothetical.

That Mn^{+3} or the enzyme system oxidizes IAA specifically at the carboxyl group seems uncertain both for reasons mentioned previously, and also because Mn^{+3} oxidizes skatole and indole, which have no carboxyl

group, in an oxygen-consuming reaction (90). However this may be, a more interesting problem is encountered with equation 7, where it is postulated that a skatoleperoxyl radical serves as oxidant for the peroxidase-catalyzed oxidation of a phenol. Extensive studies of the mechanism of peroxidase-catalyzed oxidations, especially by Chance (24), indicate that they depend on the formation of chemical complexes between peroxidase and H_2O_2 or organic hydroperoxides; there appears to be no precedent for the idea that a peroxyl radical, which is one electron more oxidized than an organic hydroperoxide, can form such a complex with peroxidase. This does not necessarily mean that it could not. The reduction of such a peroxyl radical by 1 H (equation 7) should lead, by the analogy with auto-oxidation reactions on which the sequence of reactions 6 to 7 is based (cf. 119, 146, for example) to skatolehydroperoxide ($I{\cdot}CH_2O_2H$), the type of compound which is known to be a substrate of peroxidase, might seem surprising as a product of its action, and were it formed might be capable of oxidizing two more Mn^{+2} ions, which would lead to interesting consequences. Maclachlan & Waygood (91) formulated equation 7 as indicated probably because they believed that H_2O_2 was not directly involved in the reaction; as they stated, this was not actually established, but it seems to rest largely on the observation that catalase did not inhibit. Although they did not mention how this was tested, it was probably done in the presence of one of the normally added monophenols. At least some of the "active" phenols, e.g., 2,4-dichlorophenol, inhibit the H_2O_2-destroying activity of catalase powerfully (51), and would not be a fair medium in which to test for inhibition by catalase. This question can certainly be raised with Stutz' (134) finding that catalase does not inhibit the lupine enzyme in the presence of 2,4-dichlorophenol. Resorcinol, which Waygood & Maclachlan often use, does not seem to have been tested for inhibition of catalase. These authors (147) did find that it supported the oxidation of IAA by catalase itself in the presence of Mn, and it seems possible that this would be due to the oxidation of the very phenol which results in inhibition of H_2O_2-destroying activity. At any rate, the actual oxidant in the enzymatic step would appear still to be a matter for discussion.

It might be wondered why the peroxyl radical $I{\cdot}CH_2OO{\cdot}$ would not preferentially react directly with either the phenol or the substrate, as is typical of such radicals in auto-oxidation processes, rather than with peroxidase. Maclachlan & Waygood (91) mentioned that in air, Mn^{+3} alone caused the oxidation of more than stoichiometric amounts of IAA, which they attributed to such a reaction, i.e.,

$$I{\cdot}CH_2OO{\cdot} + I{\cdot}CH_2COOH \rightarrow I{\cdot}CH_2O_2H + I{\cdot}CH_3{\cdot} + CO_2 \qquad 9.$$

Indeed, in order for the product of oxidation of IAA by Mn^{+3} to be identical with that formed in the enzymatic reaction, as these authors believe it is, some such reaction reducing most of the radicals $I.CH_2OO{\cdot}$ would have to be occurring, otherwise the product would be one equivalent more oxidized

than that formed in the enzymatic reaction, and they could not be identical. If 9 is then a rapid reaction, the main sequence would become equation 9 followed by equation 6, and the entire function of the enzyme-Mn-catalyzed cycle would be one of generating the radical $ICH_2\cdot$ as fast as it disappears by the inevitable side reactions that radicals engage in. The over-all rate would be entirely dependent on the rate of operation of the enzymatic process even though it would be merely subsidiary to the main reaction. This point illustrates the fact, which Maclachlan & Waygood have taken into consideration, that any such radical-catalyzed reaction is unstable because the radicals are unstable, and in order to maintain the reaction some process of initiation, forming new radicals, must be occurring. The kinetics of the over-all reaction are heavily dependent on the kinetics of the initiation reaction, because it establishes the concentration of radicals in the system. The importance of this to the evidence for the operation of the specific sequence of reactions 4 to 8 can be illustrated further by mentioning that Maclachlan & Waygood (91) found that in the absence of IAA, an oxygen-consuming reaction occurred involving Mn, phenol, and peroxidase; this type of reaction was postulated as the initiation reaction which generates Mn^{+3} to maintain the cycle (reactions 4 to 8). As just observed, in rate effects on the over-all reaction the kinetics of the initiation reaction will play a decisive role, and since all the constituents of the system save IAA are supposed to be involved in it, their over-all kinetic effects have to be attributed to an unknown extent to it rather than to the main reaction, in which some of them might not be involved at all even though they affect the rate. This makes rather uncertain the effort of Maclachlan & Waygood (91) to interpret the concentration effects of components of the system in terms of their probable effects on particular steps of the main sequence postulated, without first excluding their effects on the proposed initiation reaction. This difficulty will persist as long as a quantitative treatment of the initiation reaction is not found and included in a kinetic analysis of the over-all reaction. Until this is accomplished, it will be difficult to be sure whether effects on the over-all rate are even consistent with the proposed mechanism, even though it may be qualitatively argued that they are.

The specific sequence of reactions 4 to 8, thus, should not be considered as yet established; the basic idea of these authors, that a radical-catalyzed process is involved in enzymatic oxidation of IAA, is quite attractive and helps to explain many features of the reaction. It seems highly likely, from the work of Maclachlan & Waygood, that the promotive effects on Mn and phenols are connected at least in part with Mn oxidation, though it can be seen that the effect of this on the over-all rate would be just as decisive were it restricted to initiation reactions rather than being involved in the main reaction. These phenomena do not, then, exclude direct oxidation of IAA by the enzyme. Before concluding, it may be of interest to note that the idea that IAA is oxidized by peroxidase only indirectly through phenol and Mn^{+3} is analogous to the proposal of Kertesz (79) that tyrosine is not

oxidized directly by tyrosinase, but a nonenzymatic reaction with dopaquinone catalyzed by Cu^{+2}, the enzyme serving merely to reoxidize DOPA (dioxyphenylalanine) to its quinone. Although this nonenzymatic oxidation of tyrosine is demonstrable, the specificities of some tyrosinases towards oxidation of monophenols indicate that these enzymes do actually oxidize the monophenols (87, 145), and, on the other hand, it has been found (43) that side reactions of dopaquinone are so rapid compared to its reaction with tyrosine that maintained oxidation of tyrosine would be impossible if the enzyme did not act on it. The nonenzymatic process appears at best to be capable of augmenting the rate of the enzymatic one. In the case of IAA oxidation, some indication of specificity is apparent. Indole and skatole, which were oxidized by Mn^{+3} with uptake of O_2 (91), have not been observed to undergo oxygen-consuming enzymatic oxidation, and indolepropionic acid, which was also oxidized by Mn^{+3}, was not oxidized by the wheat leaf enzyme (150) or *Omphalia* enzyme (115) although it was oxidized by the lupine enzyme (134) and by horseradish peroxidase (74) when Mn was present. Lockhart (89) reported that a peroxidase preparation from *Xanthium* which did not inhibit the action of horseradish peroxidase on IAA (and had, therefore, presumably been freed of inhibitors of IAA oxidation), did not destroy IAA itself even though a phenol and H_2O_2 were present. It seems unlikely that this preparation can have been less contaminated with Mn than those highly purified enzymes which oxidize IAA in the absence of added Mn, and it may have been simply unable to react with IAA.

It is regrettable that quantitative information on the rates of oxidation of IAA and other indole derivatives by known amounts of Mn^{+3} has not as yet been published, because such information would be helpful in evaluating the actual role played by Mn^{+3} in the enzymatic reaction. Some comment seems required about the much emphasized fact that citrate and pyrophosphate, whose chelate complex with Mn^{+3} was found not to oxidize IAA (90), inhibit the enzymatic reaction, which is taken to indicate direct involvement of Mn^{+3}. These inhibitions are somewhat ambiguous, because it was found by Kenten (73) and by Stutz (134) that if concentrations of Mn as great as ordinarily employed by Waygood *et al.* (150) are present, inhibition by these agents results, even though the system may be insensitive to them in the absence of added Mn. It is possible that this inhibition could be caused by a side reaction in which the main catalytic cycle is broken by oxidation of Mn^{+2} to the inactive Mn^{+3} chelate complex, due to the much lower oxidation potential of the Mn^{+2}/Mn^{+3} system in the presence of the chelating agent (90). Once all the Mn^{+2} is oxidized, the rapid enzymatic reaction should begin; something of this kind was, indeed, observed by Kenten (73), and appears also in a curve shown by Waygood & Maclachlan (148). These inhibitions do not tell one what is happening with added Mn in the absence of the chelating agent. Maclachlan & Waygood (91) consider that the residual IAA-oxidizing activity observed with wheat leaf enzymes

in the absence of added Mn is brought about by protein-bound Mn; they also suggest that the same may be true of other IAA-oxidizing enzymes (147). The presence of such bound Mn in sufficient quantity to explain the residual activity needs to be looked for by analysis [none at all was detected in the *Omphalia* enzyme (112)]. If the supposed bound Mn is so strongly bound that citrate and pyrophosphate will not bring it off and cause inhibition, one would think it would for the same reason be unable to oxidize IAA. Otherwise, the existence of a special type of chelation amounting to formation of a "manganoperoxidase" might be suspected.

Another point which needs to be clarified is the function of maleic hydrazide in promoting IAA oxidation, which was discovered by Andreae & Andreae (6). It does not seem to have been demonstrated that this substance is a peroxidase substrate, although Waygood *et al.* (150) concluded from its effect on IAA oxidation that it must be. Kenten (73) found no evidence that peroxidase could oxidize it; that it is oxidizable seems likely from the report of Andreae (5) that it is susceptible to riboflavin-sensitized photooxidation. It can be deduced from the spectra of pyridazine derivatives given by Eichenberger *et al.* (31) that maleic hydrazide in 95 per cent ethanol exists mainly as the mono-enol, i.e., 3-hydroxy-6(1H)pyridazone, and the same is likely for aqueous solution. Thus, on paper it does resemble somewhat a monophenol; but that it acts by reacting with peroxidase remains to be shown.

Purified IAA-oxidizing enzymes.—Other than horseradish peroxidase, the only IAA-oxidizing enzyme which has been highly purified from a plant source is the enzyme from *Lupinus ulba* investigated by Stutz (134). The final step in his purification procedure involved electrophoresis; this gave a product which had peroxidase activity apparently of the order of pure horseradish peroxidase. The enzyme was considered to be electrophoretically homogeneous, but whether it was a completely pure enzyme seems uncertain considering that some of the different final preparations had rather widely differing activities. In the course of purification the ratio of IAA-oxidizing activity to peroxidase activity increased considerably; yet in the final preparation the peroxidase and IAA-oxidizing abilities had coincident electrophoretic mobilities, and almost certainly were due to one and the same enzyme. It was not established whether the change in relative activity during purification meant the removal of a peroxidase inactive in the IAA-oxidizing reaction, or the removal of inhibitors of IAA oxidation. Merely after dialysis of the homogenate, addition of a monophenol was required to give any IAA oxidation. A requirement for Mn did not appear even with the highly purified enzyme, although added Mn (10^{-3} *M*) did increase the initial rate two- to threefold, and prevented the premature decline in rate, as mentioned earlier. Stutz appears to feel that the "oxidase" and "peroxidase" activities of the enzyme may be associated with different centers on the enzyme, and he presents some interesting differences in sensi-

tivity of IAA oxidation and pyrogallol peroxidation to inhibition by certain inhibitors. He suggests that the phenol may be required to couple the "oxidase" and "peroxidase" functions by hydrogen transport, and that Mn^{+2} may promote the formation of a peroxide or radical required for oxidation of the phenol. Such possibilities could be entertained even if IAA is actually oxidized at the "peroxidase" center of the enzyme.

Polyphenols, as well as an inhibitor present in crude homogenates, caused a lengthy "lag" period before O_2 uptake began (134). Similar observations were made by Kenten (73) with horseradish peroxidase, and by Waygood *et al.* (150) with the wheat leaf enzyme. The data indicate, however, that with all the IAA-oxidizing enzymes, particularly the more purified ones, a short "lag" always precedes the rapid oxygen uptake. This has not been shown to be due to an inhibitor, and is actually what would be expected with a cyclical oxidation mechanism. It should represent the period during which the concentration of catalytic intermediates is increasing to reach the steady-state value, and is termed the "induction phase" in analogous purely chemical reactions.

The induction phase was studied (112) using the IAA-oxidizing enzyme of the fungus *Omphalia flavida,* which was discovered by Sequeira & Steeves (121) and has been described and purified by Ray & Thimann (112, 115). The kinetics of the induction phase indicated that the reaction is autocatalytic, that is, produces an excess of catalytic intermediates over what are consumed in the operation of the cycle. None of the formal schemes of IAA oxidation so far discussed has this property, although if the product of a Mn^{+3}-catalyzed enzymatic oxidation were a hydroperoxide this system would, as mentioned previously, produce a twofold potential excess of catalyst (Mn^{+3}) per turn of the cycle. With the *Omphalia* enzyme, very small amounts of added H_2O_2 completely eliminated the induction phase, and over a suitably low concentration range the initial rate depended strongly and reproducibly on H_2O_2 concentration. Estimated as its equivalent in H_2O_2, the amount of increase in catalyst even during the early stages of induction appeared to correspond to a very small fraction of the amount of IAA destroyed, so that this increase must be thought of as due to a minor side reaction rather than to the main product, as would be the case with the Mn system just mentioned. The induction phase leads into a steady-state which presumably results from rates of termination (side reactions leading to disappearance of catalyst) having become equal to the rate of initiation; the rate could be raised well above the steady-state value by adding H_2O_2 and, as would be expected, termination then exceeded initiation and the rate gradually returned to the steady-state value. The kinetics thus appear to fit a cyclical mechanism involving radicals, and are a considerable simplification because they indicate only minor dependence on initiation reactions not associated with the main IAA-destroying reaction, i.e., initiation reactions like the one suggested by Maclachlan & Waygood

(91). These authors actually stated that their reaction was autocatalytic, though they gave no evidence that this was so, and did not examine its implications for the over-all kinetics of the reaction; this possibility may be worth further study.

The most remarkable property of the *Omphalia* enzyme is that its action as a peroxidase (oxidizing pyrogallol using H_2O_2) is powerfully inhibited by IAA well within the physiological range of concentration. Inhibition of horseradish peroxidase by IAA has been reported by Siegel (125), and was confirmed (112), although much higher concentrations seemed to be required than with the *Omphalia* enzyme. This effect has not been completely explained, although it is not simply a competitive inhibition of the enzyme, because IAA oxidation is strongly inhibited at the same time. The inhibition shows specificity comparable with that of the oxygen-consuming activity of the enzyme, and since the inhibition is markedly increased in the absence of oxygen (which does not affect peroxidation in the absence of IAA), some connection with the oxygen-consuming process is suggested. The oxidation of IAA was found to be light-reversibly inhibited by CO, an observation which might suggest the formation of ferrous peroxidase, as proposed by Knox (81) for tryptophan peroxidase, and Swedin & Theorell (136) for dioxymaleic acid oxidation. However, the kinetics of this effect were peculiar (112), and it cannot be said that involvement of ferrous peroxidase was demonstrated in IAA oxidation.

Another interesting finding with the *Omphalia* enzyme was that although added Mn promoted the induction phase and steady-state rate, no promotion of the rate by Mn could be obtained when the rate was raised substantially above the steady-state by adding H_2O_2, even if a phenol were added; however, under these circumstances added phenol promoted strongly if Mn were absent. This might indicate that the phenol has some promotive effect on the main reaction independent of the presence of Mn, and that the effect of Mn may be on initiation. This is debatable, because it could be argued that the reaction mechanism may become different when H_2O_2 is added. But if this explanation is chosen then it is evident that the phenol promotes this other mechanism, which weakens the idea that promotion of the basal reaction by Kenten-Mann phenols indicates the oxidation of Mn. Promotion of the reaction by phenols under conditions where H_2O_2 was supplied was observed also by Stutz (134) and by Lockhart (89). No absolute requirement for the cofactor was demonstrated with the *Omphalia* enzyme (112), but it seems possible that its importance in the various enzymes studied may be a relative rather than absolute difference.

In conclusion, it may be interesting to return to the comparison with dioxymaleic acid oxidation originally suggested by Galston *et al.* (47). Chance (25) investigated the changes in the peroxidase spectrum which accompany this oxygen-consuming reaction; his observations suggest that something out of the ordinary, involving the not yet completely understood

H_2O_2-"Complex III" of peroxidase, was happening. He attributed the appearance of Complex III, which is normally formed only at very high H_2O_2 concentrations, to a localized high concentration of H_2O_2 near the peroxidase Fe atom as H_2O_2 is formed in the "oxidase" reaction (26). However, his formulation of this reaction as a two-electron oxidation of the substrate catalyzed by the peroxidase·H_2O_2 complex and Mn^{+2}, in which O_2 becomes reduced to H_2O_2, gives no clue as to why this reduction should actually take place, or what the role of Complex III is. Yamazaki *et al.* (159), who described a fundamentally similar oxygen-consuming oxidation of triose reductone by purified turnip peroxidase, discovered (158) that methylene blue would become reduced when the reaction was conducted anaerobically. Yamazaki showed that the kinetics of this effect support the idea that it is due to reduction of methylene blue by highly reducing radicals formed by single-electron oxidations of the substrate by peroxidase·H_2O_2. In air these radicals would, as he pointed out, be expected to react rapidly with O_2, and lead to regeneration of the primary oxidant. Interestingly enough, while Mn^{+2} promoted the oxygen-consuming reaction, it did not affect the anaerobic reduction of methylene blue, indicating that its effect lay on the interaction of the radicals with oxygen rather than on their formation, a conclusion comparable with that of Kenten (74) concerning indolepropionic acid oxidation. The implication of these findings is that, as with Maclachlan & Waygood's suggestion for IAA oxidation (91), the oxygen-consuming property of the reaction is due more to the nature of the substrate, when it becomes oxidized, than to the enzyme; there was no evidence that peroxidase transfers electrons to oxygen. However, Mason *et al.* (93) recently proposed that Complex III has a structure analogous to that of oxyhemoglobin, i.e. Fe^{+2}·O_2. No new evidence for the formation of ferrous peroxidase was presented; but the authors appear to feel that oxygen is reacting directly with peroxidase in dioxyfumaric acid oxidation. The evidence (93a) that incorporation of O^{18} accompanies hydroxylation of salicylic acid in the presence of $O_2{}^{18}$, does not seem to exclude possible reaction of an oxidized substrate radical, rather than peroxidase, with O_2. Another interesting oxygen-consuming oxidation by peroxidase is that of DPNH, reported recently by Azakawa & Conn (12). This was found to require phenols and Mn^{+2}, but the latter could be replaced by Co^{+2}. These interests suggest that a further clarification of the general mechanism of oxygen-consuming reactions catalyzed by peroxidase, and the role of Complex III, may be hoped for in the near future.

The term "dioxymaleic acid oxidase" has been in disuse for some years, since it was discovered that the "oxidase" activity in tissue extracts could be accounted for by their content of peroxidase. Oxidation of IAA may be in much the same position. It would seem that justification for the continued use of the term "IAA oxidase," implying a distinct enzyme or enzyme system for oxygen-consuming IAA oxidation in homogenates,

demands evidence on the part of the user that the observed IAA destruction or O_2 uptake must be attributed to some other enzyme than the peroxidases which these homogenates have invariably been found to contain. By analogy with tryptophan peroxidase, the more specifically active IAA-oxidizing peroxidases might be called "IAA peroxidases."

Phenolases.—Tonhazy & Pelczar (139) found that IAA was oxidized by an extracellular enzyme produced by the fungus *Polyporus versicolor,* with the same stoichiometry as in the peroxidase-catalyzed oxidations discussed above. However, they reported that no peroxidase activity could be detected in the preparations, although they must have contained powerful polyphenoloxidase activity (30). Fåhraeus & Tullander (35) discovered independently the capacity of this fungus to produce an IAA-destroying enzyme; under their culture conditions, the fungus apparently formed the enzyme in quantity only if either IAA or one of a variety of phenolic compounds were added as "inducers." Other auxins, especially indolebutyric acid, were also effective. The large rise in phenolase (laccase) activity which this results in, and the apparent absence of peroxidase (88), cause these authors to feel that the phenolase is carrying out IAA oxidation. Further study of this system should be very interesting.

Briggs & Ray (20) reported destruction of small quantities of IAA when added to a tyrosinase preparation in the presence of catechol or pyrogallol. The destruction appeared to accompany oxidation of the phenol, and perhaps represents some kind of reaction between IAA and one of the quinonoid intermediates in phenol oxidation. The reaction appeared to be a slow one, although its quantitative aspects have not been studied, but it may have some importance in disappearance of native auxin in extracts of plants which have a high phenolase and polyphenol content.

Destruction of Other Auxins

Indolepropionic and indolebutyric acids.—Enzymatic oxidation of these auxins has been referred to above; photooxidation has also been studied (40, 123). The products of these oxidations do not seem to have been examined, save for the report of Fischer (40), who considered the possibility that a compound detected in photooxidation of indolepropionic acid might be indoleacteic acid. Andreae & Good (8) found that these auxins, like IAA, are converted to aspartic acid conjugates in plant tissues; they also detected indoleacetylaspartic acid when indolebutyric acid was fed, indicating that the latter could undergo β-oxidation, as with the phenoxy acids to be mentioned below, before conjugation.

Nitriles.—The natural auxin indole-3-acetonitrile (IAN) is known to be convertible to IAA to some extent by oat (131) and wheat (120) coleoptiles, so the inactivation of IAA by known mechanisms is a possible route of destruction for this auxin. Seeley *et al.* (120) found that with wheat coleoptiles, smaller amounts of an acid which had the chromatographic

properties of indole-3-carboxylic acid were also formed from IAN, and the identity of this product was reported to be confirmed by isolation. Tissues of peas, tomato, celery, and maize were found to produce small amounts of indolecarboxylic acid from IAN, but no or only very small traces of IAA. Fawcett *et al.* (37, 38) obtained evidence of a similar type of degradation with a series of 2,4-dichlorophenoxyalkylnitriles. Wheat coleoptiles (*a*) partly transformed these to the corresponding acids by "hydrolysis" (the actual mechanism is not known), (*b*) to a lesser extent converted the nitriles by oxidation to the next lower acid (as in IAN→indolecarboxylic acid), and (*c*) degraded all the resulting acids by β-oxidation. This was deduced from the fact that when the acids themselves were fed, strict β-oxidation was observed, 2,4-D (and auxin activity) appearing only from acids with an even number of carbon atoms in the sidechain (143), whereas if the nitriles were fed, 2,4-D was formed from all members of the series, but in greater amount from even-numbered ones. This conversion of a nitrile to the next lower acid was termed "α-oxidation" (37). Curiously enough, pea stems, which did not "hydrolyze" IAN to IAA, did convert 2,4-dichlorophenoxyacetonitrile to 2,4-D, but they carried out neither "hydrolysis" nor "α-oxidation" on any of the higher members of the nitrile series, even though they did "α-oxidize" IAN. These processes thus exhibit interesting specificities. "α-Oxidation" appears to be restricted to nitriles.

Phenoxy acids.—Considerable attention has been devoted to the metabolism of the phenoxy acid auxins and analogous compounds, by soil microorganisms and by higher plants. Photooxidation is also known; Evans & Smith (34) reported detection of 2,4-dichlorophenol and glyoxylic acid, with trace amounts of glycolic acid, as products of riboflavin-sensitized photodestruction of 2,4-D. Photodestruction of 4-chlorophenoxyacetic acid was found to give 4-chlorophenol. Bell (13a) also detected formation of 2,4-dichlorophenol in riboflavin-sensitized photooxidation of 2,4-D. Phenols were formed from various phenoxyacetic acids, but not from any 3-phenoxypropionic acids, which is curious, being the reverse of what was found in flax seedlings (36, see below). Bell also studied direct ultraviolet photodestruction of 2,4-D, detecting at least 5 products on chromatograms, but no accumulation of 2,4-dichorophenol, which was attributed to the fact that this compound was itself photodestroyed by ultraviolet (why riboflavin would not photooxidize it so rapidly is not clear). Spectroscopic changes suggested that ring opening occurred to a considerable extent in dilute 2,4-D solutions, whereas in more concentrated solutions a larger proportion of ultraviolet-absorbing products, perhaps polycyclic condensates, was formed. The reported kinetics of the spectroscopic changes, though not described in detail, actually indicate the accumulation and subsequent disappearance of reaction intermediates, as with IAA photo- and enzymatic oxidation (see above).

A number of investigators have isolated, from 2,4-D-treated soil, bacteria capable of destroying 2,4-D, and in several cases there was evidence that

adaptive enzyme formation was involved. Stapp & Spicher (127b) found that 2,4-D was destroyed by an isolate named *Flavobacterium peregrinum,* giving rise to considerably more CO_2 than could be formed from the side chain alone, which suggested that oxidation of the ring occurs; considerable amounts of acid were formed, which tended to inhibit further destruction (127a). Removal of the side chain with formation of phenolic compounds also seems to be accepted as the principal type of pathway in microorganisms. Evans & Smith (34) reported that a soil microorganism which was grown on 4-chlorophenoxyacetic acid, formed the phenolic acid 2-hydroxy-4-chlorophenoxyacetic acid, and 4-chlorocatechol, as intermediates, so that unlike photodestruction, hydroxylation of the nucleus here preceded scission of the side chain. Another organism, which destroyed 2,4-D, formed a phenolic acid which the authors suggested might be 6-hydroxy-2,4-dichlorophenoxyacetic acid. Brown & McCall (22), who synthesized the various possible hydroxychlorophenoxyacetic acids, reported that Evans found the last-mentioned metabolite not to be identical with the synthetic 6-hydroxy-2,4-dichlorophenoxyacetic acid, nor with 4-hydroxy-2-chloro- nor 2-hydroxy-4-chlorophenoxyacetic acid; the identity of the latter acid with the metabolite of 4-chlorophenoxyacetic acid was, however, confirmed. Evans & Moss (33) found that the metabolism of the latter acid led beyond 4-chlorocatechol to β-chloromuconic acid, and beyond this point chloride was formed, apparently only the *cis, cis*-isomer being metabolizable.

Nuclear hydroxylation of the intact acid does not, however, seem to be the invariable mode of bacterial decomposition. Steenson & Walker (127c, 128) studied strains of *Achromobacter* and *Flavobacterium peregrinum* which after two days' culture on 2,4-D or 2-methyl-4-chlorophenoxyacetic acid would oxidize several micromoles of the acid to CO_2, water and HCl in a few hours. Organisms adapted to these acids would also oxidize the analogous bromo derivatives and also 4-chlorophenoxyacetic acid. 2,4-dichlorophenol and 4-chlorocatechol were also rapidly oxidized, whereas 6-hydroxy-2,4-dichlorophenoxyacetic acid and 3,5-dichlorocatechol were not. According to the criterion of simultaneous adaptation, it thus appeared that formation of 2,4-dichlorophenol directly from 2,4-D was the pathway utilized, and probably thence by hydrolysis of the 2-chloro-group giving 4-chlorocatechol. It does not seem to be explained why the observed rapid oxygen uptakes with 2,4-dichlorophenol and 4-chlorocatechol ceased far short of complete conversion to CO_2, water and HCl, and with the latter substance led to accumulation of a yellow, possibly quinonoid, substance, whereas the acids, as previously mentioned, were completely and rapidly oxidized; the toxic effects which were found to be exerted by the phenols may be involved. Formation of the yellow substance was, however, observed with 4-chlorophenoxyacetic acid and 4-chlorophenol, in which case 2-hydroxy-4-chlorophenoxyacetic acid was not attacked, suggesting direct cleavage here also, followed by hydroxylation.

The capacity of 2,4-D-destroying organisms to oxidize 2,4-dichlorophenol rapidly has also been established by Rogoff & Reid (118a), with a strain of *Corynebacterium,* and by Walker & Newman (143a), with an organism identified as a *Mycoplana.* The latter organism, however, was reported also to oxidize rapidly 2-hydroxy-4-chlorophenoxyacetic acid, as well as 4-chlorophenoxyacetic acid. These authors (143a) obtained from soil a variety of other isolates on 2,4-D-enriched media, but several of these could not be propagated indefinitely, perhaps owing to complex nutritional requirements. Bell (13b) isolated an organism, tentatively placed in *Achromobacter,* which would thrive only on complex media, but used 2,4-D as a principal energy source. He found that under certain conditions 2,4-dichlorophenol accumulated in the medium. With the above organisms it was shown that nearly quantitative oxidation of added 2,4-D (13b, 118a, 143a), and liberation of its Cl as chloride (13b, 118a), occurred.

Audus & Symonds (10) reported some curious observations on 2,4-D inactivation in cultures of a *Bacterium globiforme* organism. Disappearance of phytotoxicity was followed with a cress-root inhibition assay, and it appeared that the organisms completed their growth in a few days and had long since ceased to multiply while phytotoxicity remained practically constant; then, in a matter of one or two days, toxicity disappeared entirely. The authors felt that conversion of 2,4-D to some phytotoxic intermediate, followed by adaptation to metabolize the intermediate, must be occurring, though why the latter would not be accompanied by further growth is unclear. The assay was probably not specific for auxin activity and might have responded to possible phenolic intermediates such as 2,4-dichlorophenol, which are known to inhibit growth (15).

Inactivation of 2,4-D in bean plants was recently followed with the pea-curvature auxin assay by Hay & Thimann (59). When 2,4-D was introduced at a flap cut in one of the primary leaves of the seedling, most of the 2,4-D which was translocated out into the leaf and beyond became inactivated in four days (none got beyond the hypocotyl region), whereas the 2,4-D remaining at the point of application persisted, as if the mechanism for inactivation either did not exist there or had become disrupted. Light stimulated inactivation. The results appear to be in rather good agreement with earlier quantitative work of Jaworski & Butts (68) using C^{14}-labelled 2,4-D, in which the labelled hormone in extracts was estimated, after chromatography, by its radioactivity. The interesting difference between this inactivation and the microbial processes mentioned above is that it seems to involve complete degradation of the molecule, or even of the side-chain, to a minor extent. The data of Weintraub *et al.* (153) indicate that by the time the applied 2,4-D has been inactivated, less than about 7 per cent of the label from carboxy-labelled 2,4-D, and less than 2 percent from methylene-labelled 2,4-D, has appeared as CO_2; later release of CO_2 is even slower. This is apparently explained by the finding of Jaworski *et al.*

(68, 69) that the labelled hormone is largely transformed into two water-soluble substances, the more abundant of which was said to be hydrolyzable to give 2,4-D, and may have been some kind of conjugate. Holley (65) reported that a seemingly similar derivative gave, when hydrolyzed, an acid different from 2,4-D, which he suggested might be a hydroxy-2,4-dichlorophenoxyacetic acid, i.e., similar to some of the compounds mentioned in microbial metabolism of 2,4-D. The nature of the conjugate seems to remain unclarified, a glycoside of a phenolic acid being possible, or perhaps a derivative analogous to indoleacetylaspartic acid. Andreae & Good (8) did not detect formation of such a derivative, but the inactivation of applied 2,4-D appears to be a relatively slow process in plants compared with that of IAA, just as is said to be the case in soil (109). 2,4-D was recovered entirely unchanged after 3 hours' uptake by oat coleoptiles (71). It can persist in some plants for months (58, 154). Free 2,4-D has been shown to accumulate in plants fed even-chain 2,4-dichlorophenoxyalkylcarboxylic acids (38), whereas IAA did not accumulate when indolebutyric acid was fed (8). From the foregoing it is understandable that phenoxyacetic acids do not appear to be converted to a major extent to phenols in the higher plants which have been studied. Fawcett *et al.* (36) observed that substantial amounts of phenol were formed by β-oxidation only from phenoxyalkylcarboxylic acids with an odd number of C atoms in the side chain. The terminal steps in β-oxidation of such an acid were formulated as formation of the β-keto derivative of phenoxypropionic acid (equivalent to the phenyl monoester of malonic acid), followed by conversion to O-carboxyphenol, then decarboxylation to phenol. It also seems possible that direct hydrolysis of the malonic acid ester might take place; this type of reaction was advanced to explain the formation of considerable amounts of phenol from phenoxydecanoic acid, where ω-oxidation, which is known in animals, would form the phenyl monoester of decandioic acid. β-Oxidation of the even-chain phenoxyacetic acids stops at the acetic derivative, which is converted to phenol much more slowly than the propionic derivative; small amounts of phenol were, however detected (36).

With analogous ω-substituted alkylcarboxylic acids, interesting combinations of β-oxidation with nuclear hydroxylation have been observed recently by Webley *et al.* (151) with *Nocardia opaca,* and by Byrde *et al.* (23) with *Aspergillus niger;* Webley *et al.* (152) have detected a β-hydroxy intermediate in the conversion of 4-chlorophenoxybutyric to 4-chlorophenoxyacetic acid by the former microorganism.

Physiology of Auxin Destruction

Aside from the relationship to phytotoxicity of herbicides, which is not covered in this review, the principle areas in which auxin destruction has been suggested to be of physiological importance are in phototropism, in control of growth, in ageing of tissues and in certain pathological effects.

The relation of auxin destruction to phototropism will not be reviewed here.

The idea that over-all growth of plants can be influenced by auxin inactivation goes back to some of the early work on auxins, in which the existence of particularly active peroxidase or auxin destruction was noticed with dwarf varieties of plants. This notion might appear to be largely supplanted by the finding that the growth of many dwarf varieties is retarded because of apparent deficiency in a hormone of the gibberellin type, which is not known to affect auxin inactivation. [However, some kind of relation between auxin inactivation and gibberellin was recently suggested by Vlitos & Meudt (141a)]. Von Abrams (2) compared auxin inactivation in one dwarf and a closely related normal variety of peas, and found there to be no substantial difference, even though treatment with auxin would accelerate the growth of the dwarf more than that of the normal. The possibility that some cases of dwarfing may be due to excessive auxin-destroying activity cannot, however, be said to be ruled out, especially as gibberellin is not effective with every dwarf strain. The idea was recently revived by Kamerbeek (72), who made quantitative measurements of peroxidase activity in sister normal and dwarf bean plants derived from hybridizations, and found some cases of much greater activity in the dwarfs. Galston (46) also recently reported markedly greater auxin-destroying activity in a dwarf variety of pea.

Control of elongation.—Galston and his collaborators have developed the thesis that destruction of auxin limits the zone of elongation in stems and roots, and promotes "ageing" or maturation of plant cells. This was first suggested by Galston & Dalberg (48), and was based primarily on the finding that a gradient of increasing IAA-destroying activity appeared with sections of pea stem or root increasingly distant from the apex. It was suggested that towards the lower end of the elongation zone, the rapidly increasing rate of auxin destruction causes the auxin concentration to fall rapidly to the point where growth ceases, and cell maturation occurs. It could be objected that, as the authors were no doubt aware, the appearance of the "gradient" depended on the use of protein N as a basis for expressing activity, no gradient or a reverse gradient being found on a fresh-weight basis. However, it is certain that the protoplasm is not being largely diluted with water during elongation, most of the increase in fresh weight representing water taken into the vacuole. Since auxin transport is a vital phenomenon and hardly would be expected to take place elsewhere than in the protoplasm, the concentration of destroying enzyme in the protoplasm, for which a protein N basis should be satisfactory, may be taken as the basis for calculating activity, as these authors have done. In any case, it would seem to be essential that comparison between diffusible auxin content and auxin-destroying activity be made on the same basis, whether weight or N.

Galston & Dalberg (48) also discovered that treatment of sections of

stems or roots with solutions of IAA well within the (for stems) physiological concentration range produces substantial increases in IAA-destroying activity when homogenates are subsequently tested. They considered this an adaptive synthesis of the IAA-oxidizing enzyme; it would seem to be remarkably similar to the adaptive increase in tryptophan peroxidase which Knox & Mehler (83) found to occur when tryptophan is administered to animals. Galston & Dalberg attributed considerable significance to this phenomenon, suggesting, among other things, that the timing of it could be a factor in the appearance of the gradient in auxin-destroying activity and, consequently, the limiting of the elongation zone.

Pilet & Galston (106) and Pilet (96, 99) demonstrated in roots of *Lens culinaris* a gradient in IAA-destroying activity having a minimum at the apical meristem and increasing both towards the root cap and through the zone of elongation. This activity was reported (100) to be close to inversely correlated with the chloroform-extractable auxin content of the same sections of the root. IAA-destroying activity was also found to show an inverse correlation with the -SH content of the tissues, which may be interesting in view of the report of Sharpensteen, Galston & Siegel (122) on inhibitory effects of some -SH compounds on peroxidase. The rate of "peroxide" formation (106), measured with the pyrogallol assay mentioned earlier (127), showed a rough correlation with the IAA-destroying activity; both of these were strongly promoted by added phenols in the meristem region, and only little affected in the most mature regions. This suggested that the increase in activity back of the meristem might be due to a gradient in concentration of a natural cofactor analogous in action to the monophenols; the occurrence of such natural factors has been mentioned previously. In pea shoots, longitudinal gradients also in respect to the naturally occurring inhibitors of IAA destruction have been reported by Galston (46). Henderson (60) reported a gradient in auxin-destroying activity along the length of the *Avena* coleoptile.

Jensen (70) found that peroxidase activity in the zone of elongation of *Vicia faba* roots was strikingly increased by previous treatment with IAA. This effect might be responsible for the inductive action of IAA on IAA-destroying activity since, as discussed earlier, IAA-destroying activity in homogenates appears to be attributable to peroxidase. However, it is hard to see how the IAA-destroying activity gradient in *Lens* roots could be the result of this kind of induction, if the gradient is due to changes in amount of cofactor; nor in peas, if it involves changes in amount of inhibitor.

The increase in peroxidase induced in *Vicia* roots by treatment with IAA (70) occurred mainly in the provascular tissue, a finding that is in harmony with the evidence [Siegel (124, 126)] that lignification in vascular tissue involves the action of peroxidase, and with the fact that treatment with auxin accelerates vascular differentiation in shoots and roots (67, 140). The question may be asked whether the inductive effect of IAA on

IAA-destroying activity in tissues may not be a consequence of its stimulating the beginning of vascular differentiation, which involves peroxidase formation, rather than being an adaptative enzyme effect in the usual sense. This might explain why other auxins, which are neither substrates nor inhibitors of IAA-oxidizing enzymes, "induce" IAA-destroying activity (48). The differentiation of some specialized tissues may also involve the accumulation of phenolic substances (42) which are likely to act either as cofactors for or inhibitors of IAA oxidation. It is an evident possibility that the entire gradient in IAA destroying activity may be simply a consequence of the anatomical changes which take place in the course of development of the stem or root, and might have nothing directly to do with auxin inactivation in the plant.

These points illustrate the fact that the observed inverse correlations between activity and auxin content or growth are, as is usually recognized, only correlations and are not necessarily causally connected. Since auxin is synthesized largely at the apex of the shoot or root, it is also easy to imagine control of the zone of elongation by an auxin-inactivating system which would not change in activity along the length of the organ, but which would, for example, destroy a constant fraction of the auxin passing through each unit of length. The correlations and inductive effects can in this way be seen not to be essential conditions for control of growth by auxin inactivation. In fact, it would be easier to explain the numerous effects of translocated auxin far beyond the zone of elongation, not to mention the demonstrable presence of translocated auxin there, if auxin-destroying activity decreased as one went beyond the zone of elongation, instead of increasing further as appears in some of the data. What seems, then, to be needed is some convincing indication that systems do operate within the plant to destroy its own auxin, and equally important, that growth at the base of the elongation zone ceases because the auxin concentration falls too low. The frequently-made observation that the portion of the axis below the elongation zone will not elongate when auxin is supplied is wholly irrelevant, since these tissues have undergone anatomical changes which of themselves virtually prevent elongation. Before concluding with a dicussion of whether auxin destruction occurs within the plant or not, other aspects of physiology which have recently been related to IAA destruction will be considered.

Other possible effects.—Pilet (97) reported that segments of carrot root exhibited much higher IAA-destroying activity at the basal than at the apical end, the end which more readily forms callus; in sterile culture the activity of the old tissue rose still further, while it remained low in the growing callus. It was thought that this might reflect an aspect of the ageing of nongrowing tissues relative to the growing one. A similar relationship was considered for *Lens* roots, by comparing the meristem with the adjacent root cap (102). Pilet & Baillaud (105) reported a possible relationship

between auxin-destroying activity and nutation in bean stems. Konishi (84) suggested a connection between IAA-destroying activity and growth form in stems of *Silene armeria* in transformation of the rosette to the elongating stage.

Henderson, Miller & Deese (62) reported that high concentrations of 2,4-D inhibted IAA destruction by oat coleoptiles and promoted it with pea and sunflower stem sections, an effect which they thought might be related to the selective action of 2,4-D. They also published *Avena*-test results which might indicate that endogenous auxin in the latter plants, but not in oats, was greatly diminished after 2,4-D treatment (61). Audus & Thresh (11, 138) found no depressing effect of 2,4-D on auxin content in peas, sunflower, or beans. They also reported that no decrease in auxin content resulted from treating peas with maleic hydrazide. Somewhat similar findings with maleic hydrazide were made with tissue cultures by Kulescha (85, 86), and with *Lens* roots by Pilet (98). These observations are of interest because, as mentioned above, maleic hydrazide stimulates the activity of IAA-oxidizing enzymes, and it had originally been thought reasonable (4, 6) that its inhibitory effect on growth might result from this property, an idea which now does not seem to be maintainable. Pilet (103) also showed that strong inhibitions of *Lens* root growth were caused by maleic hydrazide at concentrations several orders of magnitude lower than are required to bring about promotion of IAA oxidation in homogenates of the same material. Concentrations of maleic hydrazide high enough to actually promote destruction in extracts do not seem to have been used on plants. Brian & Hemming (18a) recently suggested that the inhibitory action of maleic hydrazide on growth is due to its antagonizing "gibberellin-like" hormones in plants.

The promotive effects of Mn on IAA destruction, discussed above, have prompted some investigations of the possible role of Mn nutrition on auxin-destroying activity in plants. Winfree (157) stated that high Mn nutrition increased IAA-destroying activity of soybean, whereas Fujiwara & Tsutsumi (45) found that barley plants under severe Mn (or Fe!) deficiency showed no diminished IAA-destroying activity in root extracts, whereas Cu or Mo deficiency caused pronounced decrease in activity. How reliable these assays were seems uncertain. With reference to Mn^{+2} oxidation, Kenten & Mann (78) found that oxidized forms of Mn accumulated in pea plants supplied with toxic amounts of Mn^{+2}.

Sequeira & Steeves (121) suggested that defoliation caused by the parasitic fungus *Omphalia flavida* could be due to inactivation of auxin within the parasitized leaf by an extracellular enzyme produced by the fungus. Pilet (104) reported that another leaf-parasite, *Uromyces pisi,* caused an increase in auxin content in parasitized leaves of *Euphorbia,* which could be attributed to formation by the fungus of substances which inhibited the auxin-destroying enzymes of the leaf.

An interesting effect on IAA destruction recently studied by Hillman & Galston (64) is the inhibition of activity which results if dark-grown plants are given red light some hours before an enzyme extract is prepared; this effect was reversible by far red in a fashion similar to that of many other red light effects. It was shown to be due to formation of dialysable inhibitors of IAA oxidation as a result of red light treatment. Such endogenous inhibitors have been mentioned in the foregoing discussion; it had been noticed in the original work of Tang & Bonner (137) that the inhibitors were much more abundant in light-grown plants; these inhibitors are not always easy to remove, and often mask the IAA-destroying activity in green tissues.

Auxin destruction in vivo.—It has been known for many years that the yield of natural auxin obtainable from some plants by diffusion or in homogenates is decreased by the action of destroying enzymes which, if they operated equally rapidly in the intact plant, might seem to preclude the existence of auxin in the plants at all. Recently, Åberg & Jönsson (1) obtained evidence that IAA destruction by pea roots was activated by sectioning. Steeves *et al.* (129) found that application of a drop of 0.005 *M* KCN to the cut ends of fronds of *Osmunda cinnamomea* in the agar-block diffusion technique considerably increased the yield of auxin, sometimes no auxin at all being obtained without cyanide, presumably because of the operation of inactivating enzymes at the cut surface. They subsequently showed (19) the occurrence of enzymatic inactivation of IAA in homogenates of these fronds, and concluded further [Briggs *et al.* (21)] that this inactivation is probably an "artifact" which occurs only with cut or damaged tissue, and could not be expected to play a role in the control of leaf growth. It was found that although some auxin seems to disappear in the zone of elongation, no substantial decrease in auxin yield by diffusion occurred through the entire length of the zone of maturation (several cm.). In two experiments (19, 21) IAA destruction by extracts of the leaf apex (crozier), elongation zone and zone of maturation was compared, and was found to decrease markedly in activity in the order given, no activity at all being detected in the zone of maturation in the more complete experiment, and negligible activity, judging from the apparent precision of the assay, in the other experiment. In this latter experiment (21) diffusion data were also obtained from the same fronds, before homogenization, at points between each of the zones and below the last, with the usual result that some auxin disappeared in the elongation zone but none did in the zone of maturation. As far as these two zones are concerned, the data would appear to indicate a good correlation between *in vivo* auxin disappearance and activity of the extracts. As previously mentioned, however, the authors actually felt that the extracted system was an artifact, and it seems probable that they must have observed much more rapid IAA destruction in other extracts of the zone of maturation. They also found, in a separate diffusion experiment,

that the auxin yield did not decrease through a section of the rachis within the crozier, extracts of which, as just indicated, contained the greatest IAA-destroying activity; but since the extracts were of the whole crozier rather than this particular segment of rachis, it is difficult to compare the two. To return to the zone of elongation, it is interesting that some 25 per cent of the initially added IAA was destroyed in 5 hours by the homogenate (diluted 3-fold from the intact tissue), while 40-70 per cent of the auxin estimated to enter this zone ***in vivo*** seemed to disappear in transit through the zone, a process which appears also to take about 5 hours; it might seem that the activity in the extracts is only barely adequate to account for the *in vivo* destruction. However, the IAA concentration in the extract experiments appears to have been about 100 times greater than within the plants (calculated as IAA from the diffusion and extract data provided), so it is difficult to compare the two results realistically without knowledge of the reaction kinetics, which is not available. The authors felt the auxin disappearance in the elongation zone was due instead to some other mechanism and somehow associated with growth, an idea based on the observation of Bonner & Thimann (16) that growth in decapitated *Avena* coleoptiles was approximately in proportion to auxin disappearance in them. An actual connection between auxin destruction and the growth mechanism does not seem to have been as yet demonstrated, the data of Bonner & Thimann (16) indicating that auxin disappears in nongrowing coleoptiles also. In any event, it is difficult to see how one could ever decide whether auxin destruction is controlling growth, if the auxin destruction which occurs in the growing zone is to be expressly excluded from consideration (cf. 21), for, as previously indicated, this is the only region in which the question can be tested.

These comments may indicate the many uncertainties which beset the effort to relate IAA destroying activity in homogenates to processes going on in the intact plant. The work of Briggs *et al.* is a valuably critical approach to this problem, and it makes plain the need for further direct evidence on rates of destruction of native auxin in intact plants, which it may be hoped will receive more attention in the future.

Fang and Butts (35a) found that $C^{14}O_2$ was produced when carboxyl-labelled IAA was applied to bean, pea and maize plants. Such a release of $C^{14}O_2$ would be expected to accompany destruction of IAA by the enzymatic oxidation mechanisms and by photooxidation. With all the plants, $C^{14}O_2$ production was much greater in light than in darkness; with maize no $C^{14}O_2$ was detected in the dark. Whether this means that photooxidation is important, or is due to promotive effects of light on enzymatic oxidation [cf. (47, 56, 147)] or other causes, is not clear. In light, a considerable proportion of the applied C^{14} appears to have been recovered as $C^{14}O_2$ within one day. Since the IAA solutions were injected into the leaves, and most of the radioactivity which stayed in the plants remained in the region of applica-

tion, an unknown amount of the $C^{14}O_2$ formation may have been due to IAA destruction in injured tissues, so one cannot feel sure the results are representative of oxidation of IAA in the intact plant. The authors (35a) found that 2,4-D depressed the formation of $C^{14}O_2$ from labelled IAA. This seems to be the reverse of the effect reported by Henderson *et al.* (61, 62), mentioned above.

Reinhold (117) attempted to evaluate the importance of the cut surface in IAA disappearance from solutions containing sections of pea stems, by comparing the rate of disappearance using whole sections and sections divided into a number of smaller pieces. The apparent fraction of the total uptake associated with the cut surface amounted to about one-seventh with 3 cm.-long sections. In these experiments no IAA could be recovered from the tissue, all of what entered having been either bound or destroyed. A similar result was obtained by Andreae & van Ysselstein (9), who studied simultaneously the disappearance of IAA from solution and the accumulation of indoleacetylaspartic acid and IAA within pea stem sections. At externally applied concentrations below 10^{-4} *M* IAA, very little IAA accumulated within, and indoleacetylaspartic acid equivalent only to about one-fifth of the IAA disappearing was recovered, the remaining IAA being unaccounted for and presumably either bound in some way or, more likely, destroyed. Since these authors were using sections over 3 cm. long, on the basis of Reinhold's (117) results most of this destruction would have to be attributed to intact tissue. At IAA concentrations above 10^{-4} *M*, free IAA did accumulate inside the tissue, and it was found, surprisingly, that if such tissue were removed to a moist chamber, the IAA within was nearly quantitatively transformed into indoleacetylaspartic acid, no other type of destruction apparently occurring. As the authors (9) suggested, this might indicate that IAA destruction was not occurring in all tissues of the stem, but occurring so rapidly in those where it does take place, that no free IAA can accumulate; the tissues where free IAA accumulates would be those which only have the capacity to conjugate it. This idea is supported by the finding that the concentration of applied IAA at which IAA begins to accumulate within, is the same as that at which the rate of indoleacetylaspartic acid formation becomes maximal (about 8×10^{-5} *M*). Another explanation might be that most of the IAA destruction with externally applied IAA takes place as it passes into the tissue, say in the epidermis, and that once in the cortical or pith parenchyma it is subject mainly to conjugation. In any case, a considerable degree of localization of IAA-destroying activity of intact tissue is implied. This conclusion would be in harmony with the evidence that IAA destruction in homogenates is largely attributable to peroxidase, because peroxidase is one of the best known of histologically localized enzymes, being found mainly in the vascular tissue and epidermis, as the extensive review of van Fleet (41) indicates.

The notion that IAA-destroying activity may be largely absent from par-

enchymatous tissues such as cortex or pith would pose grave problems for its significance in controlling elongation, since it is well known that in some organs, e.g., the *Avena* coleoptile, auxin is actively transported in parenchyma, and it is generally believed that at the auxin concentrations which occur within the elongation zone, lateral movement takes place to a minor extent, else tropisms could not be explained. The possibility that physiologically meaningless measurements of activity in homogenates may result from the mixing of enzyme, phenols, and inactive protein from different tissues, is also serious. However, it could also be argued that the localized occurrence of destroying enzymes and of potent inhibitors and stimulators of their activity could permit rapid destruction of excessive amounts of auxin while allowing gentle action on the hormone under normal circumstances. Despite the unreasonably high activity of some extracted preparations, there would seem to be considerable room for further inquiry into the actual role of these enzymes in the physiology of plants.

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METABOLISM OF ASCORBIC ACID IN PLANTS: PART I. FUNCTION[1,2,3]

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Introduction

The metabolism of ascorbic acid in plants may, broadly speaking, be divided into two main fields. They are (*a*) the biological systems concerned with the synthesis of the vitamin, and (*b*) its function in the plant cell after synthesis. The function of the vitamin in plants is discussed in this review, and it is hoped that a review dealing with biosynthesis will follow in a later volume. The biochemical pattern of many fundamental metabolic functions is very similar in plants and animals; and for this reason no apology is made for including in this review work which has been carried out on animals or animal tissues, for any knowledge from whatever source it originates may be of value in future studies.

Since the distribution and location of ascorbic acid within the plant cell has some bearing on its synthesis and function, it is convenient to discuss this aspect before dealing separately with either.

Distribution and Location in Plants

Distribution.—All primates are dependent on plant sources for their supply of the antiscorbutic factor; its occurrence and distribution within the plant kingdom is, therefore, a subject of general interest. Ascorbic acid occurs as such and in its oxidised form, dehydroascorbic acid, in nearly all plant tissues; but its concentration within different tissues of the same plant, or in different plants, varies over a wide range. Generally, the green leafy portions of plants contain it in highest concentration with lesser amounts in the petals, stems, or roots. It is not proposed to give here an extended range of values for ascorbic acid in plant tissues since these have been adequately tabulated elsewhere [Fixsen & Roscoe (1); Platt (2); Daniel & Munsell (3); Olliver (4)]. These values for any particular fruit or vegetable are, of course, average values, and in spite of the wide limits within which their ascorbic

[1] The survey of literature pertaining to this review was concluded in August, 1957.

[2] This review was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research.

[3] The following abbreviations will be used: AA, ascorbic acid; ATP, adenosine triphosphate; DDC, diethyldithiocarbamate; DHA, dehydroascorbic acid; DPN, diphosphopyridine nucleotide; DPNH, diphosphopyridine nucleotide (reduced form); FMN, flavin mononucleotide; GSSG, oxidised glutathione; GSH, reduced glutathione; TPN, triphosphopyridine nucleotide; TPNH, triphosphopyridine nucleotide (reduced form).

acid content might be expected to vary, do show reasonably good agreement in plants grown in different localities and under different conditions. From such data it is quite clear that the level of the vitamin in different plants varies enormously. Thus among the fruits, plums appear to exist quite normally with 0.03 mg. ascorbic acid/gram fresh tissue, whereas black currants contain 70 times and unripe walnut fruits 600 to 700 times as much. At present no simple reason can be given for this somewhat haphazard distribution, other than the obvious one that the concentration of ascorbic acid as found represents the difference between rate of synthesis and rate of utilization in metabolism, and since at present we have no exact means of measuring these rates, it must remain one of conjecture.

Within the single plant, ascorbic acid is mainly concentrated in regions of high metabolic activity; thus, Reid found it to occur in the greatest concentration in the actively growing zones, particularly in regions undergoing elongation, in leaf buds, and in floral buds (5). The same author (6) found that in root tips the transition from the embryonic to the elongating phase was associated with a sevenfold increase in the ascorbic acid content, and she made the interesting suggestion that this accumulation preceded and conditioned cell elongation.

Bound ascorbic acid.—Ascorbic acid has been alleged to occur in plant tissues combined with protein as "ascorbigen" (7) and has been the subject of numerous investigations. The reader is referred to the review by Chayen (8) for information on the earlier work. There is recent evidence showing that ascorbic acid occurs bound to protein in liver tissue, and can be separated from the free form by its insolubility in 95 per cent alcohol. Ascorbic acid may be liberated from the protein complex by treatment with acids [Sumerwell & Sealock (9)]. These results were confirmed by Dayton *et al.* (10)using C^{14}-labelled L-ascorbic acid. About 19 per cent of the total liver ascorbic acid was present in the bound form; D-ascorbic acid when given to the animal was also isolated in the bound form. While there can be no doubt that some ascorbic acid in an insoluble form can be found in liver tissue after extraction with ethanol, the possibility that treatment with ethanol produced the compound was not excluded. There is no comparable evidence to show whether ascorbic acid is bound to protein in plant tissue; Procházka (10a), however, has isolated a bound ascorbic acid from cabbage (10b), which has been shown (10b) to be a compound in which the dienol group of ascorbic acid is combined with an indole derivative, indoleacetic acid (10c). This compound, which has been found only in the Brassicaceae, has no known physiological function (10d).

Intracellular location.—Numerous attempts have been made to determine the location of ascorbic acid within the cell, and its ability to reduce silver nitrate in the cold has usually been employed for this purpose [Bourne (11, 12); Giroud *et al.* (13); Mirimanoff (14)]. The reagent, although less specific for ascorbic acid than other reagents, possesses the advantage that the end product, reduced silver, is insoluble, is deposited, and can be seen

against the background of the surrounding cytoplasm. Barnett & Bourne's (15) technique is based on the fact that although many cell components will reduce silver nitrate, few will reduce acidified silver nitrate; the tissue is, therefore, fixed and impregnated with acidified silver nitrate solution. Modifications of this technique have been devised by Chayen *et al.* (16).

Giroud *et al.* (17) and Mirimanoff (18), in the hope that some light might be thrown on either the site of its synthesis or of its function within the cell, attempted to identify the chloroplasts as the site and chlorophyll as the necessary agent for its synthesis, on the evidence that when plant tissues are treated with silver nitrate the reduced silver often appears localized within the chloroplast. This has been the subject of investigation by several workers. In *Gladiolus*, known to be rich in ascorbic acid, a blackening of the reticulum of the chloroplast was observed and in *Polygonatum multiflorum* a blackening of the chloroplasts was usually observed [Weber (19)]. These results have usually been interpreted as indicating the localization of ascorbic acid within the chloroplast, the chief criticism being that the reduction of silver nitrate is by no means specific for ascorbic acid. In fact, Gautheret (20) claimed that the reduction of the silver salt by the chloroplasts was caused by chlorophyll acting as an optical sensitizer, since he found that the most effective wavelength of light for the reduction of silver salts by the chloroplasts coincided with the absorption spectrum of chlorophyll. Nagai (21), however, has reinvestigated this question using modern methods of chromatographic analysis, and has found ascorbic acid to be the most commonly occurring substance in plant tissues capable of reducing silver nitrate in the cold at neutral or acid pH. He did, however, identify dihydroxyphenylalanine (DOPA) as a constituent of some tissues which reduced silver nitrate under these conditions. Although the agent responsible for the reduction of silver nitrate can frequently be shown to be ascorbic acid, Nagai (21) found that the localized deposition of silver in the chloroplasts was due to the migration of colloidal particles from other parts of the cell. His conclusions, as far as plant tissues are concerned, are very similar to those of Reiner (22) for animal cells, where the specificity of the agent for ascorbic acid was found to be reasonably good, although no deduction based on the localized deposition of silver particles could be made owing to diffusion phenomena.

Chayen *et al.* (16) found that in both bean and onion root tips the meristem region contained the heaviest concentration of precipitated silver. In the meristematic cells, silver deposits occurred in the cytoplasm but were in greatest abundance on the chromosome region of interphase nuclei and on mitotic chromosomes. The authors point out that there are three stages during treatment of the tissue at which diffusion of substances may occur which make it difficult to determine the localization of ascorbic acid; these are: (*a*) artefacts attendant on chemical fixation, (*b*) diffusion of ascorbic acid after fixation, and (*c*) movement of silver grains from the site at which they were formed. Treatment to eliminate such artefacts are discussed by

Chayen (8) who supplemented the cytochemical techniques with a spectrophotometric method whereby the loss of absorption at 276 mμ after treatment with copper salts was used to determine the presence of ascorbic acid. These studies again showed ascorbic acid to be present on the chromosomes as well as in the cytoplasm, but this interpretation was again questioned since mitochondria were destroyed during the fixation process.

Other techniques [Zirkle (23); Newcomer (24)] have been used to preserve cytoplasmic structures, and with these silver deposits were seen on granules resembling mitochondria; this agreed with Bourne's findings (11, 12) with embryonic animal cells. It seems likely that the deposition of silver grains on chromosomes is an artefact and is due to diffusion of the vitamin from the mitochondrial particles when these structures are destoyed by the fixation process.

The histochemical evidence available at present gives no clear indication as to whether ascorbic acid is concentrated in any one element of the cell. Certainly it seems to be present in the particulate fractions of the cell for the mitochondrial elements have been implicated as the site of synthesis [Mapson *et al.* (25)]. However, there is no reason to suppose that the vitamin is exclusively localized within these particles for it has been found in the soluble fraction of the cytoplasm after their removal [Mapson & Moustafa (26)], and in the cell nuclei [Stern & Timonen (27)].

Ascorbic Acid and Respiration—Oxidising Systems

The discovery of ascorbic acid came at a time when biochemists were becoming increasingly interested in enzymes and substrates concerned with oxidation. Into this world the discovery of ascorbic acid appeared to fit exceedingly well. It was a substance known to be necessary for animal life, and was known to exist in an oxidised and reduced form even before its constitution was finally identified. Furthermore, its unusual distribution in plant tissues, together with the fact that its occurrence coincides quite generally with high metabolic activity, and its ease of oxidation by enzymes in plant tissues, first demonstrated by Szent-Györgyi (28), seemed to assure for it a role as an electron carrier *in vivo*.

It may not be out of place in the consideration of ascorbic acid as a respiratory carrier to enumerate the properties that a substance must possess in order that it may function as such *in vivo* (29).

1. The substance must be a natural component of tissues.
2. The substance must be capable of being reduced by tissues at a rate comparable with the rate of oxidation of substances whose oxidation it is presumed to catalyse.
3. The reduced compound must be capable of being oxidised by the tissues at an adequate rate.
4. The substance must be capable of stimulating the rate of hydrogen transfer in the system under investigation.

5. The compound must be directly reduced by one system and directly oxidised by a second system which is not identical with the first.

Ascorbic acid, as will be seen in the following review, satisfies many of these requirements, although the studies in which all these criteria have been demonstrated are few.

Enzymes catalysing the oxidation of ascorbic acid.—Ascorbic acid is not autoxidisable within the physiological range of pH, so that catalysts are essential for any reaction between it and oxygen. If, therefore, it plays any physiological role in respiration, one would naturally expect to find enzymes capable of catalysing its oxidation.

Many such enzyme systems have been found in plant extracts, and these may be subdivided into two categories: (*a*) those in which the oxidation of ascorbic acid is secondary to the oxidation of the substrate of the enzyme, and (*b*) those in which there is a direct reaction between enzyme, ascorbic acid, and oxygen. In the first group are included polyphenolase, laccase, cytochrome oxidase, and peroxidase. In the latter group there is, as far as our knowledge goes, only one enzyme, ascorbic acid oxidase, that occurs in the higher plants. An atypical ascorbic acid oxidase has, however, been found to occur in *Myrothecium verrucaria* by Mandels (30) and a similar enzyme in *Physarum polycephalum* by Ward (31). These enzymes differ from the oxidase found in the higher plants in that (*a*) their activity is not inhibited by copper chelating agents, e.g., cyanide, 8-hydroxyquinoline, or diethyldithiocarbamate (DDC), (*b*) H_2O_2 is produced in the enzymic oxidation of ascorbic acid, and (*c*) they are specific for L-ascorbic acid and will not oxidise, D-arabo-ascorbic, or D-gluco-ascorbic acid. The nature of the prosthetic group in these enzymes is as yet unknown.

All of the known enzymes in higher plants capable of oxidising ascorbic acid are proteins containing either Cu or Fe as the prosthetic group and all of them could be responsible for the entry of oxygen into the respiratory system of plants. None of them can function in the absence of O_2 or in the presence of cyanide, hence it follows that any system in which ascorbic acid is acting as a respiratory carrier will be inoperative in the presence of cyanide. The properties of these enzymes have been recently reviewed by Bonner (32) and will not, therefore, be repeated here.

It is clear that the oxidation of ascorbic acid may be catalysed by any or all of the oxidase systems occurring in plants, but such biochemical studies furnish little evidence as to what extent these reactions proceed *in vivo*. All these oxidases carry oxidation only as far as the dehydroascorbic acid stage. The fact that ascorbic acid appears to be associated in fresh tissues with small but definite amounts of dehydroascorbic acid, combined with the probability that dehydroascorbic acid is continually being lost by irreversible conversion to 2,3-diketogulonic acid, makes it appear probable that there is a continuous oxidation of ascorbic acid *in vivo*. Support for this may be found in the work of Wood *et al.* (33) and Barker & Mapson (34) in

which it was shown that the normal loss of ascorbic acid from leaves or potato tubers may be prevented under anaerobic conditions, combined with the fact that under such circumstances the level of dehydroascorbic acid in the cell may reach zero values [Mapson & Barker (35)].

Oxidase activity as modified by substances occurring in plants.—It seems clear that the full potential effect of many of the oxidase enzymes capable of oxidising ascorbic acid is not realised *in vivo.* Thus, for example, the polyphenolase activity of extracts from potato tissue is equivalent to an electron transfer some 100 times greater than the total respiration of the intact tuber, [Mapson & Barker (36)] and the ascorbic acid oxidase of pea stem internodes is capable of consuming oxygen at a rate 40 times as great as the total respiration of the tissue [Eichenberger & Thimann (37)]. To account for this, many reasons have been advanced such as (*a*) spatial separation of substrate and enzyme, (*b*) substrate in a form not acted upon by the enzyme, and (*c*) the presence in tissue of substances modifying the action of the enzymes.

There are references in the literature to the inhibition of oxidase enzyme by naturally occurring substances. The presence of a substance in many fruits and vegetables which effectively inhibits the oxidation of ascorbic acid, whether this is brought about by ascorbic acid oxidase, polyphenolase, peroxidase, or by inorganic Cu, has been reported by Somogyi (38), but the nature of this substance or substances was not identified.

Damodaran & Nair (39) isolated a tannin from the Indian gooseberry (*Phyllanthus emblica*) which inhibited the oxidation of ascorbic acid in the press juice. Since the protective effect of this substance could be "overridden" by the addition of Cu, they concluded that its action depended on the suppression of metal catalysis.

Kardo-Sysoeva & Nisenbaum (40) reported a thermolabile stabiliser for ascorbic acid in the tomato but gave no indication of how it functioned. Giri & Krishnamurthy (41) likewise separated a substance from the juices of *Cucumis sativus, Cucurbita maxima* and *Luffa acutangula,* which prevented the oxidation of ascorbic acid even in the presence of Cu.

The presence of volatile constituents which are alleged to inhibit the enzymic destruction of ascorbic acid in cabbage and other green vegetables has also been reported [Brand (42)]. Hooper & Ayres (43) have found that black currants, a fruit in which ascorbic acid is remarkably stable, contain substances which inhibit the oxidation of ascorbic acid by the polyphenolase system of apples. The protective action was found to be associated with a red pigment (anthocyanin fraction) and with a yellow pigment ("flavanone"). Similar red pigments in beet roots also gave marked protection against the oxidase of apple juice. These substances from the black currant afforded no protection, however, against the enzymatic oxidation of ascorbic acid by ascorbic oxidase from cucumber juice; they were thus distinct from those of Somogyi. Extracts containing vitamin P were also tested but with negative results. The results of Somogyi and of Brand are more readily inter-

preted as a direct effect on the enzyme, for in Somogyi's experiments, his substances inhibited the action of ascorbic oxidase and polyphenolase as well as that of inorganic copper, and in Brand's, the probability seems high that his volatile constituents from cabbage contained sulphydryl derivatives which have high chelating powers for copper. The experimental results of Hooper & Ayres are more likely to be due to competitive inhibition, produced by the high concentration of glycosidic flavonoid substances present in their extracts. The protection they observed against polyphenolase but not against ascorbic oxidase would then be explicable.

Some idea of the many possibilities whereby the activity of a copper enzyme such as ascorbic acid oxidase may be modified by the many substances present in plants is obtained from a study of the more recent work with this enzyme. Inactivation of the enzyme during the progress of the oxidation of ascorbic acid has been noted by Dawson & Tarpley (44) and Singer & Kearney (45). Powers & Dawson (46) explained the inactivation of ascorbic acid oxidase as resulting from two causes: (*a*) inactivation due to environmental conditions during the reaction, protection against which was afforded by inert proteins such as egg albumen and gelatin, and (*b*) inactivation due to some factor inherent in the ascorbic acid-ascorbic acid oxidase reaction sensitive only to haem containing proteins, such as catalase or peroxidase. Frieden (47) showed that the activating and protective influence of thyroxine on the enzyme reported by Gemmill (48) was not specific but was shown by any effective cupric ion complexers such as ethylenediamine tetraacetic acid (EDTA), cyanide, diethyldithiocarbamate (DDC), cysteine, and other amino acids. Chemical compounds which activate or protect the enzyme, or both, could be divided into two groups: (*a*) those which activate or protect at all concentrations, and (*b*) those which protect at low concentrations but inhibit at higher concentrations. The first group include EDTA, cysteine, nucleic acid, nucleotides, and proteins and the second is comprised of cyanide, DDC, and 8-hydroxyquinoline. These latter stimulate the activity at levels of 10^{-6}, 10^{-7} *M* but inhibit at concentrations of 10^{-4} *M* or higher. Frieden and co-workers (49, 50) observed that the enzyme was sensitive to organic mercurials, to Cu^{++} and Hg^{++} salts, and that the resulting inhibition could be reversed by either cysteine or reduced glutathione (GSH), which led them to suggest that it was a sulphydryl dependent enzyme.

To explain these observations Joselow & Dawson (51) and Frieden & Maggiolo (52) have put forward very similar suggestions. The former workers, having shown that during the oxidation of ascorbic acid there is an exchange of radioactive Cu ion with that of the enzyme, suggested that such Cu ions might normally be liberated during the reaction and be effective in catalysing the oxidation of adjacent thiol groups of the enzyme. This would account for the progressive inactivation of the enzyme during the reaction and the high toxicity of added Cu ions. Frieden & Maggiolo consider that a free radical intermediate of ascorbate formed during the reaction may accept

electrons from the thiol groups of the enzyme, which are thereby inactivated. Protection of the enzyme might result where a copper ion complexer protects adjacent thiol groups by its interaction with the enzyme. Finally the complete inhibition that results, with some copper chelating agents in high concentration, may arise by the removal of copper from the enzyme protein.

Dehydroascorbic acid in plant tissues.—In his earlier experiments, Szent-Györgyi (53) found that ascorbic acid was rapidly oxidised on the addition of hydrogen peroxide but that, on standing, the ascorbic acid was regenerated. This latter observation could not be repeated if the juice was boiled. Since that date evidence has accumulated that ascorbic acid and dehydroascorbic acid (DHA) are interconvertible in plant tissues. In some fruits, notably apples, the proportion of DHA to that of ascorbic acid is high in the early stages of development but decreases as the fruit approaches maturity [Zilva *et al.* (54)]. The direct formation of ascorbic acid from DHA was demonstrated in cabbage [Rubin *et al.* (55)] and in poplar leaves [Mapson & Barker (56)] after DHA had been injected or fed to these tissues. The cyclic oxidation and reduction of ascorbic acid under aerobic and anaerobic conditions has also been observed in sucrose/phosphate extracts from pea cotyledons [Mapson (57)].

The concentration of DHA in plant tissue is usually small in relation to that of ascorbic acid; published data indicate that in fresh tissue it is of the order of 5 per cent or less of the total ascorbic acid content. Ascorbic acid and DHA are usually estimated after extraction of tissue by acids such as metaphosphoric or oxalic. This procedure is normally carried out at room temperature, precautions being taken to disintegrate the tissue as rapidly as possible under the surface of the extractant (58). Indeed it was the failure to do this efficiently which caused the earlier workers, Ahmad (59) and McHenry & Graham (60), to conclude that some of the ascorbic acid existed in bound form, since more ascorbic acid could be estimated if the tissue was heated beforehand. These results were shown to be due to a more rapid inactivation of oxidase enzymes by heat than by the faulty techniques employed for extraction in the cold [Fujita & Ebihara (61); Wacholder & Kreut (62)].

New and more specific methods for the estimation of DHA [Mapson & Ingram (63); Hughes (64)] have made it possible to reinvestigate the conditions necessary for the estimation and determination of ascorbic acid and DHA in tissue. Work by Barker & Mapson (65) has shown that even when plant tissues are disintegrated as rapidly as possible in extractants such as metaphosphoric acid at room temperature, inactivation of oxidase enzymes is not fast enough to prevent some oxidation of ascorbic acid. Using extraction procedures at temperatures ranging from $-3°$ to $-70°$C. and taking precautions to exclude oxygen from all solutions before and during extraction, the concentration of DHA in relation to total ascorbic acid in a number of plant tissues has been found to be much smaller (2 to 10 times smaller) than that found after using acid extraction methods at room temperature.

Although it has been found impossible to obtain a conclusive answer as to whether even this residual amount of DHA is to be considered as representing the true value of this constituent in the tissue *in vivo*, it is perhaps significant that this small amount of DHA was always found unless the tissue had been placed for some hours in nitrogen previously.

It is the writer's belief that there is a small amount of DHA present in cells, and it is equally clear that normally the cell maintains most of its ascorbic acid in the reduced state. This balance in the plant cell may be disturbed by a number of factors such as :(*a*) mechanical damage, (*b*) action of substances causing cellular disorganization (e.g., narcotics), or (*c*) by the action of specific enzymic poisons, *e.g.*, iodoacetate, arsenite, or fluoride (66). Under such conditions there is a rapid conversion of ascorbic acid to DHA. Various reasons have been advanced to explain the phenomenon. On the one hand, there is the hypothesis that damage to the cell causes disorganisation and allows the oxidases to come into more effective contact with their substrates, resulting in a greatly increased rate of oxidation. A second explanation is that the enzymic systems concerned with the reduction of ascorbic acid are impaired. A third explanation that on mechanical damage the oxygen tension in the tissues is raised seems improbable in view of experiments which show that raising the oxygen tension from that of air to that of pure oxygen has no immediate effect on the balance of ascorbic acid-DHA in the intact cell [Mapson & Barker (66); Barker & Mapson (67)]. Whichever explanation is correct, it seems certain that reducing systems are present in plant tissues which maintain ascorbic acid in the reduced form. The failure to discover such reducing systems was originally an obstacle to the acceptance of the view that ascorbic acid may act as a respiratory catalyst. In the past too much attention may have been paid to the fact that ascorbic acid occurs in the plant in the reduced form and to its effect as a hydrogen donator. The fact that ascorbic acid accumulates in the cell in the reduced form may simply mean that the systems which reduce it are more active *in vivo* than are those which oxidize it. What indeed may be of greater significance is the concentration of DHA or of other intermediate oxidation products of ascorbic acid (98), which by reason of their ability to accept hydrogen may determine the level at which the ascorbic acid system acts as a respiratory catalyst.

There has, to the present date, been no comprehensive attempt to determine whether there is any correlation between the concentration of DHA and the level of respiration in plant tissues. Some attempts have been made to correlate the level of respiration of certain plant tissues with their ascorbic acid content. Franke (67a) observed that the increase in the respiration (CO_2 production) of germinating seeds of *Sinapis alba*, and of slices of potato tissue ran parallel with an increase in the ascorbic acid of the tissue. Rubin *et al.* (68) found that with the fruit of two species of *Rosa*, *R. cinnamonea* and *R. spinosissima*, both the level of respiration and the ascorbic acid were higher in the former than in the latter species. An increase of ascorbic acid

of the fruit, induced by the infiltration of glucose, increased the respiration. However, this correlation was not observed during ripening, for here the level of respiration rose but the concentration of ascorbic acid decreased. The failure to observe a complete correlation under all conditions was explained by the hypothesis that only a part of the total ascorbic acid was participating. This argument gains some support from the fact that the concentration of ascorbic acid in some plant tissues may be very high, which makes it difficult to believe that it is all functioning as a respiratory catalyst. The level of ascorbic acid may in fact be altered in the plant cell without having any significant effect on the respiratory level. For instance, it was observed [Chen & Mapson (69)] that cress seedlings, grown under conditions where the amount of ascorbic acid synthesized varied greatly, did not show any difference in their general level of respiratory activity. We must conclude that such studies have so far given little positive evidence either for or against ascorbic acid functioning as a respiratory catalyst.

If the DHA-ascorbic acid system is acting as a respiratory carrier *in vivo* then one would expect that the subjection of plant tissues to anaerobic conditions would lead to a fall in concentration, if not to the complete disappearance of DHA. An analogous phenomenon is certainly observed with cytochrome in portions of intact potato tissue. The reduction of cytochrome-*c* under anaerobic conditions and its re-oxidation on admittance of air may be observed spectroscopically by visual examination of the cytochrome spectrum of the tubers *in vivo* [Hill & Scarisbrick (70)]. Moreover, these changes are produced within 60 to 90 min. of the alteration of the atmosphere around the tubers [Hill & Barker (71)]. There are reasons which make it difficult to draw firm conclusions from a similar analysis of the reduced and oxidised forms of ascorbic acid. Thus, it may be argued that despite precautions taken to exclude oxygen during extraction at low temperatures, the removal of oxygen from a tissue placed in an oxygen-free atmosphere would of itself prevent oxidation of ascorbic acid, and hence the lower values of DHA found could be explained on these grounds alone.

Recent experiments [Mapson & Barker (35)] with strawberry and cabbage leaves have shown that there is a rapid and progressive decline in the concentration of DHA on placing the tissues in nitrogen. With strawberry leaves the concentration of DHA falls to a zero value within about 15 min. at 20°C.; with cabbage, a low value but no zero values have been observed. The progressive nature of the fall suggests that it is due to reduction of DHA rather than to prevention of oxidation during extraction.

The fact that a zero value for DHA was not obtained with cabbage leaves may be connected with the observation that if the tissue is left in nitrogen for periods greater than 30 min. there is a progressive increase in the concentration of DHA. The same phenomenon which has been observed with potatoes and strawberry leaves, and is as yet unexplained, makes it difficult to be sure that the rate of disappearance of DHA, as measured after short periods in nitrogen, represents the true rate of its reduction. Assuming, however, that this measurement gives some indication of the rate of

reduction *in vivo*, results of preliminary experiments indicate that the electron transfer over DHA was equivalent to from 10 to 30 per cent of the total respiration of cabbage or strawberry leaves. In similar work, Mapson & Moustafa (26) measured the reduction of DHA by homogenates prepared from pea cotyledons and came to the conclusion that the rate of this reaction could account for about 20 per cent of the total respiration of the cotyledons. Admittedly on these estimates the proportion of the respiration mediated via ascorbic acid is small, but it is by no means negligible.

Ascorbic acid oxidase as a terminal oxidase.—All the known enzymic systems which have been suggested as governing the entry of oxygen into the plant cell catalyse the direct or indirect oxidation of ascorbic acid, and of these the available evidence suggests that the cytochrome system is the most important. Although negative evidence of the participation of ascorbic acid oxidase as a terminal oxidase would not exclude the possibility of ascorbic acid functioning as an electron carrier *in vivo*, positive evidence would indicate that it does in view of the fact that ascorbic acid is the only known natural substrate for this enzyme.

It needs to be emphasized that the ability of a tissue or tissue extract to oxidise ascorbic acid is not a sufficient criterion for the presence in that tissue of a specific ascorbic acid oxidase. This may seem obvious, but in many cases cited in the literature this has been the only criterion used. Hallaway & Butt (72) have recently drawn attention to those criteria which may be applied to determine whether the observed catalytic oxidation of ascorbic acid is attributable to a specific enzyme or to catalysis by Cu^{++} or by nonspecific Cu proteinates. These are (*a*) a determination of the turnover number (moles ascorbic acid oxidised/atom Cu/min.), (*b*) the order of reaction (Zero order for ascorbic acid oxidase, first order for Cu^{++} catalysis) and (*c*) effect of EDTA (negative with ascorbic acid oxidase, complete inhibition with Cu^{++} catalysis).

Except for the indirect evidence of Waygood (73) ascorbic acid oxidase has usually been considered a soluble enzyme. Stafford (74), using aqueous extract of pea seedlings, found the enzyme in the soluble fraction (not sedimented at 60,000 g), and Mapson & Moustafa (26), using a sucrose/phosphate extraction medium, also located the enzyme in the soluble fraction. Similar results to these have been reported by Eichenberger & Thimann (37) for pea stem internodes, and by Nason *et al.* (75) for tobacco leaflets. It is clear, however, that in some tissues the enzyme is associated with cell-wall material; Newcomb (76) found it to be confined to the cell wall in tobacco-stem pith cultures, and Honda (77), likewise, found that the major part of the enzyme in barley roots was associated with cell-wall fragments, although the amount of the enzyme found in the soluble and mitochondrial fractions was sufficient to account for the respiration of the tissue. The nature of the extraction medium seems also to have some bearing on this problem for Hallaway & Butt (72) have found that the inclusion of sucrose in the extracting solution increases the proportion of soluble enzyme.

The claim that ascorbic acid oxidase was a terminal oxidase in the respira-

tion of pea seedlings and apples was made both by Davison (78) and Hackney (79) on the somewhat inadequate ground that (*a*) the enzyme was present, and (*b*) that the respiration was stimulated by the addition of ascorbic acid. Waygood (73) working with wheat seedlings came to the same conclusion for similar reasons with the added evidence that polyphenolase enzymes were absent, and that cytochrome oxidase could only be detected in the embryonic stage.

More positive evidence of the functioning of ascorbic acid oxidase as a terminal oxidase comes from the work of James and his colleagues (80, 81). They found that of the three oxidases, ascorbic acid oxidase, cytochrome oxidase, and polyphenolase, only the first could be demonstrated in 10 to 17-day-old barley roots. During the development of these roots there appeared to be a gradual replacement of cytochrome oxidase by ascorbic acid oxidase, as evidenced by the fall in sensitivity of the respiration to CO and a rise in sensitivity towards sodium diethyldithiocarbamate (DDC) which pointed to a progressive change from an iron catalysed to a copper catalysed system.

A fall in sensitivity of the respiration to CO may of itself merely indicate that there is a change from a CO sensitive cytochrome system to an insensitive one [Bendall & Hill (82)], and in addition there is still some doubt about the sensitivity of ascorbic acid oxidase to CO. James (83) states that the enzyme in barley is insensitive, and Eichenberger & Thimann (37) reached the same conclusion for the oxidation of ascorbic acid, as catalysed by ex-extracts from pea stem internodes. On the other hand, Honda (84) observed a 20 per cent inhibition of ascorbic acid oxidation by CO with intact barley roots, and Matsukawa (85) and Diemair & Zerban (86) report inhibitions with the extracted enzyme. The oxidation of ascorbate by the marrow enzyme was inhibited 20 to 25 per cent by 95 per cent CO: 5 per cent O_2 at 10°, and 10 to 14 per cent at 30°; these inhibitions which were not light reversible, were not reduced with gas mixtures, containing 80 per cent CO: 20 per cent O_2. The corresponding oxidation of ascorbate by ionic Cu is completely inhibited with 95 per cent CO: 5 per cent O_2 [Hallaway & Butt (72)].

Other workers have found cytochrome oxidase in barley roots (87, 88), and Honda (89) found it in 10 to 12-day-old barley roots in a concentration sufficient to account for the respiration of the tissue. He has emphasized the difficulties involved in making an assessment of the role of oxidase based simply on the effect of inhibitors on respiration. He obtained evidence that DDC besides inhibiting ascorbic acid oxidase had an inhibitory effect on succinic dehydrogenase *in vivo*. His failure to obtain a quantitative relationship between the inhibition of ascorbic acid oxidase and of respiration by different inhibitors does not of itself prove that the enzyme is not acting as a terminal oxidase, especially since in the tissue studied most of the enzyme was localized within the cell walls. Thus if, as seems likely, the contribution of the cell-wall material to the total respiration was small, complete inhibition of this would have little effect upon the total respiration. Honda, in

fact, suggests that the inhibition of the oxidase without a corresponding inhibition of respiration by weak solutions of phenyl thiourea may well have been due to inactivation of the surface localized enzyme, but not of the enzyme present in the cytoplasm. Similarly, the marked inhibition of the respiration of root segments without a comparable inhibition of the ascorbic acid oxidase after pretreatment with high concentrations of the inhibitor (10^{-2} *M*) and washing, may have been caused by the removal of the inhibitor from the surface layers by washing without removing it from the cytoplasm, thereby reversing the inhibition of the enzyme in the surface but not in the cytoplasm. Such results emphasize the difficulties attendant on the use of inhibitors for determining the role of enzymes in intact tissue. Hallaway & Butt (72) have recently reinvestigated the oxidation of ascorbate by barley root segments. Judged by their criteria the oxidation is catalysed by a specific oxidase and appears to take place at the root surface. There was no indication that the ascorbate entered into the metabolism of the tissue since (*a*) there was an equivalence of O_2 uptake and ascorbate supplied and the total ascorbate (ascorbic acid+dehydroascorbic acid) in the external solution remained unchanged during the oxidation, and (*b*) the endogenous oxygen uptake and CO_2 production was unaltered. Moreover, the oxidation of ascorbate could be completely inhibited by 50 μM DDC, without reducing the endogenous respiration by more than 25 per cent. In addition, the ability of different 1 cm. root segments taken along the length of eight day roots, to oxidise ascorbic acid remained constant, whereas the respiration of the segments progressively fell.

These results appear to exclude any role for the surface localized ascorbic acid oxidase in barley root respiration. However, it was also found that the surface activity represents only 25 to 40 per cent of the total oxidase activity found in a root homogenate, although all of this oxidase was precipitated with the cell debris fraction. Such results while of interest in relation to the surface localized oxidase, give no indication of whether any of the "inactive oxidase" found in homogenates was active ***in vivo***.

On the basis of inhibitor studies, Király & Farkas (90) reached the conclusion that cytochrome oxidase, not ascorbic acid oxidase, was the main terminal oxidase in healthy wheat leaves, but that in leaves infected with stem rust, the enhanced respiratory activity became highly sensitive to copper chelating agents, and was paralleled by an increase in ascorbic acid oxidase activity. The authors suggest that ascorbic acid oxidase may be present in healthy plants in an inactive state but only becomes operative in infected plants and is the terminal oxidase of the parasitically stimulated respiration. Changes in terminal oxidation under the influence of vernalization (91), illumination (92), and parasitic attack (93) have been reported by other workers.

Eichenberger & Thimann (37) found an active ascorbic acid oxidase in pea stem internodes from etiolated seedlings, which was unaffected by CO. The intact tissue consumed oxygen at a rate only one-fortieth as great as

that for which the ascorbic acid oxidase could account. However, since 80 per cent of the oxygen consumption was inhibited by CO and was light reversible, the authors concluded that cytochrome oxidase was the main terminal oxidase and considered it probable that ascorbic acid oxidase was either produced during the preparation of the extracts or was nonfunctional in the intact cells.

Thimann and colleagues (94) have sought to establish that ascorbic acid oxidase is not in many tissues of major importance as a terminal oxidase on the basis of the low affinity of the enzyme for oxygen (half maximal activity at a partial pressure of O_2 of 16 per cent), which compares with an oxygen partial pressure of only 0.5 per cent required by potato discs and 5 per cent for pea stem internodes for half maximal respiration. The findings with the oxidase (a commercial sample) would have been more conclusive had the criteria suggested by Hallaway & Butt been used to show that the catalytic effect was in fact due entirely to a specific ascorbic acid oxidase. However, Thimann's results have now been substantially confirmed by Hallaway & Butt (72) working with a highly purified marrow enzyme; a decrease in partial pressure of O_2 from 20 per cent to 5 per cent led to a decrease of 65 per cent in the rate of oxidation of ascorbate.

Bonner's (32) conclusion that Thimann's results eliminate ascorbic acid oxidase as a terminal oxidase in respiring tissues, would only be true if the oxidase catalysed the rate limiting step at all oxygen tensions, and this seems unlikely in view of our knowledge that the ATP turnover probably governs this.

The evidence reviewed here still leaves it uncertain whether ascorbic acid oxidase ever functions as a terminal oxidase. Although most observers would agree that the major terminal oxidase in plants generally is cytochrome oxidase the evidence does not exclude the possibility that in some tissues at different stages of development or under certain environmental or pathological conditions part of the respiration may be mediated via ascorbic acid oxidase.

Ascorbic Acid and Respiration—Reducing Systems

Reducing systems linked with diphosphopyridine nucleotide (DPN).—The early work of James & Cragg (95) first suggested a link between certain dehydrogenase systems and ascorbic acid. Having demonstrated the existence of an ascorbic acid oxidase in barley seedings, they found that three acids, viz., glycolic, lactic, and tartaric, increased the oxygen consumption in the presence of ascorbic acid but not in its absence; other acids, including citric, ascorbic, malic, acetic, pyruvic, and succinic, were without effect. The increased oxygen consumption with the former group of acids was due ultimately to their oxidation and not to the oxidation of ascorbic acid, since little or no loss of ascorbic acid occurred. In the case of lactic acid the formation of pyruvic acid was shown. In further experiments (96) it was shown that with barley saps to which hexose diphosphate and ascorbic acid were

added, an increased oxygen uptake occurred in excess of that caused by the addition of ascorbic acid alone. This oxygen uptake could be still further increased by the addition of DPN. The breakdown of hexose diphosphate to phosphoglyceric acid was stimulated by the addition of ascorbic acid. The course of hydrogen transport was, therefore, believed to be triose phosphate→DPN→ascorbic acid→O_2.

Results of a similar character were obtained by Davison (97) in pea seeds and pea seedlings. These tissues contained an active formic dehydrogenase which reduced dyes such as Nile blue in the presence of DPN; the reduced dye could be reoxidized by DHA. The oxygen consumption of such tissue was increased when formate was added and still further increased on the addition of ascorbic acid.

Further evidence of like character in support of the participation of DPN in reactions associated with the reduction of DHA has been advanced by Waygood (73). Cell-free extracts of wheat seedlings were found to contain a malic dehydrogenase enzyme, reducing DPN, as well as ascorbic acid oxidase and peroxidase enzymes. When to such extracts malic acid, DPN, and ascorbic acid were added, together with a fixative for the oxalacetate formed in the reaction, the system absorbed oxygen in excess of that required for the complete oxidation of ascorbic acid. In this system methylene blue could replace ascorbic acid.

Other substrates such as fumarate, alcohol, or hexose diphosphate were also found to give similar results. The increased oxygen consumption in most of these experiments was greater than 1 mole of oxygen per mole of ascorbic acid added, i.e., greater than that required for the complete oxidation of ascorbic acid to oxalic and threonic acid. This fact, combined with the finding of small amounts of dehydroascorbic acid at the end of the experiment (13 per cent of the original amount of ascorbic acid added), was taken to indicate that the oxygen consumption observed was due to formation of DPNH, which reduced dehydroascorbic acid and was itself reduced in the presence of the dehydrogenase enzyme and substrate. In an incomplete system (no DPN added), neither ascorbic nor dehydroascorbic acid could be detected.

Mathews (98) showed the presence in peas of an enzyme which catalysed the oxidation of DPNH by oxygen in the presence of either ascorbic acid or methylene blue. These reactions were not inhibited by cyanide. The enzyme preparation also catalysed the oxidation of triphosphopyridine nucleotide (TPN) with methylene blue, but not with ascorbic acid. The most interesting feature of these experiments was the demonstration that DHA did not act as a hydrogen acceptor. Mathews suggested that the acceptor was a semiquinone intermediate, a monodehydroascorbic acid formed by the metal catalysed oxidation of ascorbic acid.

Beevers (99) likewise observed the enzymic oxidation of DPNH upon the addition of ascorbic acid to a cucumber extract containing ascorbic acid oxidase. Nason *et al.* (100) and Kern & Racker (101) have extracted and

purified the enzyme from pea seeds and yeast and have shown that oxygen and a catalyst for the oxidation of ascorbic acid, whether it be ascorbic acid oxidase or a metal such as Cu^{++} or Fe^{+++}, are necessary for the reaction. The electron acceptor was not DHA nor could it be formed by the nonenzymic reduction of DHA by glutathione or cysteine. Only oxidizing catalysts undergoing single electron changes were effective in forming the acceptor substrates. The labile nature of the acceptor was demonstrated; within 10 min. at room temperature most of the acceptor had been converted into DHA. Kern & Racker purified the enzyme from yeast and found it to be a flavo-protein; the apoenzyme formed by splitting off the prosthetic group could be reactivated by the addition of either riboflavin phosphate (FMN) or by flavin-adenine-dinucloetide (FAD). D-arabo-ascorbic acid and D-gluco-ascorbic acid could both replace L-ascorbic even with purified enzyme preparations, but TPNH could not be substituted for DPNH with the purified yeast enzyme, but some activity towards TPNH was observed with the pea enzyme.

Confirmatory evidence of the existence of such an enzyme and of its participation in an electron transfer system in animal tissues comes from the work of Kersten *et al.* (102) who found that ascorbic acid catalyses electron transport between DPNH and the cytochrome system in extracts from the adrenal glands of pigs. In whole homogenates DHA was partially effective but in a mitochondrial preparation only ascorbic acid was effective.

The existence of a system whereby DPNH may be linked with ascorbic acid and from thence to molecular oxygen appears therefore to be well established for both plant and animal tissues. Presumably the system could function through either ascorbic acid oxidase or cytochrome oxidase, although the latter possibility has not been demonstrated in plants. To what extent such a respiratory pathway is operative *in vivo* remains to be determined.

Reducing systems linked with triphosphopyridine nucleotide (TPN).—(*a*) Dehydroascorbic acid reductase: The protective effect of glutathione (GSH) on the oxidation of ascorbic acid has been observed by several workers, who have, however, explained it in different ways. The fact that GSH combines readily with copper was believed by some workers to be the explanation. That this is not the sole reason is shown by the fact that GSH will reduce dehydroascorbic acid in solution above pH 6.5 without added catalysts [Borsook *et al.* (103); Bukin (104); Yamaguchi & Joslyn (105)]. Following Szent-Györgyi, many authors have ascribed the power of tissues or tissue extracts to reduce DHA to their content of GSH [de Caro & Giani (106); Mawson (107); Barron *et al.* (108); Borsook *et al.* (103); Rubin *et al.* (55)].

The rate of the uncatalysed reaction is, however, too slow to be of much consequence, the half-time period for the reduction of dehydroascorbic acid by GSH at physiological temperatures and at pH values and in concentrations usually found *in vivo* being of the order of 15 min., whereas under the same conditions the conversion of DHA to 2,3-dioxo-L-gulonic acid has a half life of only 2 min. [Ball (109)].

Pfankuch (110) was the first to describe enzymatic reduction of DHA by sulphydryl compounds. He found an enzyme in potato juice which catalysed the reduction of DHA by cysteine. This work was extended by Hopkins and his collaborators [Hopkins & Morgan (111, 112); Crook & Hopkins (113); Crook & Morgan (114)] who showed that an enzyme, dehydroascorbic acid reductase, catalysed the reduction of DHA by GSH in accordance with the following equation:

$$2GSH + DHA \rightleftharpoons GSSG + AA$$

The enzyme was prepared from cauliflower juice and separated from ascorbic acid oxidase. The distribution of the enzyme was investigated, and it was found to be present in 22 of the 30 species of plants examined, the most active sources being broad beans and cauliflowers. Yamaguchi & Joslyn (105) who purified the enzyme found it in peas, particularly in the meristematic regions were the respiration rate is high. There is still some doubt as to the specificity of the enzyme for the hydrogen donor. Crook & Morgan (114) working with an enzyme prepared from cauliflower reported that cysteine and thiolactic acid could replace GSH whereas Yamaguchi & Joslyn (105) found the enzyme from peas to be specific for the hydrogen donor GSH, but less specific for the acceptor; both dehydro-D-araboascorbic acid and 1,2,3 triketo cyclopentane were reduced but at a much slower rate than dehydro-L-ascorbic acid.

The possibility that this enzyme is one stage in a hydrogen transfer system was suggested by Crook (116) who visualized the system

$$\text{plant substrates} \rightarrow \overset{H}{\text{—S—S—}} \rightarrow DHA \rightarrow O_2$$

a suggestion which was criticised by Barron (115) and answered by Crook (116).

(*b*) Glutathione reductase: The ability of plant tissues to reduce -S-S- compounds to SH compounds was evident from the early work of Kohman & Sanborn (117) and Ganapathy (118). Hopkins & Morgan (111) in their studies on the sulphydryl compounds produced during the germination of pea seeds, established that the chief compound concerned was reduced glutathione (GSH), which was rapidly produced from oxidised glutathione (GSSG) if this compound was added to extracts of the seeds.

The work was extended by the observations of Mapson & Goddard (119) and Conn & Vennesland (120), working with peas and wheat, respectively, who showed the presence of an enzyme, glutathione reductase, in these tissues. This enzyme catalyses the reduction of GSSG by TPNH. The enzyme appears to be highly specific for GSSG, for it will not catalyse the reduction of cystine, homocystine, α-glutamyl cystine, or aspartathione. It is also specific for TPNH; there is no reaction with DPNH. Anderson *et al.* (121) found the enzyme to be widely distributed in plants. Nickerson & Romano (122) have found a similar enzyme cystine reductase to be present in yeast and

peas; this enzyme is, however, specific for both cystine and DPNH, and is thus distinct from glutathione reductase. The claim by Bukin (104) that GSSG may be reduced nonenzymatically by DPN or TPN has not been substantiated (119).

(*c*) Glutathione-Ascorbic acid system: Szent-Györgyi (123) first suggested on the basis of the known behaviour of ascorbic acid that it might, in conjunction with glutathione, act as a respiratory carrier. The following hypothetical reactions were visualised as occurring:

1. $DHA + GSH \rightarrow AA + GSSG$
2. $GSSG + \text{glucose phosphate} \rightarrow GSH + CO_2 + H_2O$
3. $AA + O_2 \xrightarrow{\text{ascorbic oxidase}} DHA + H_2O_2$
4. $\text{Flavone} + H_2O_2 \xrightarrow{\text{peroxidase}} \text{Flavone oxide} + H_2O$
5. $\text{Flavone oxide} + AA \rightarrow \text{Flavone} + DHA$

With the discovery of glutathione reductase, an electron transferring system involving TPN, GSH and ascorbic acid was at once apparent. Such a system was in fact demonstrated in pea seed extracts by Mapson & Goddard (119). Extracts from this tissue contain both malate and isocitrate dehydrogenase enzymes which reduce TPN, together with glutathione and dehydroascorbic acid reducatase, and it was shown that, as a result, hydrogen from isocitrate or malate may be transferred to dehydroascorbic acid in accordance with the following reactions.

$$\begin{matrix}\text{Isocitrate} \\ \text{Malate}\end{matrix} + TPN \rightleftharpoons TPNH + \begin{matrix}\text{Oxalosuccinate} \\ \text{Oxalacetate}\end{matrix}$$

$$\begin{matrix}\text{Oxalosuccinate} \\ \text{Oxalacetate}\end{matrix} \xrightleftharpoons{\text{Mn and decarboxylase}} CO_2 + \begin{matrix}\alpha\text{-Ketoglutarate} \\ \text{Pyruvate}\end{matrix}$$

$$TPNH + GSSG \xrightleftharpoons{\text{glutathione reductase}} 2GSH + TPN$$

$$GSH + DHA \xrightleftharpoons{\text{dehydroascorbic acid reductase}} AA + GSSG$$

In an extension of this work with pea seedlings Mapson & Moustafa (26) were able to reconstitute a respiratory system whereby hydrogen was transferred from substrates of TPN linked dehydrogenases to molecular oxygen. The enzymes concerned in the respiratory pathway included dehydrogenase enzymes (malic and isocitrate enzymes), glutathione reductase, dehydroascorbic acid reductase, and ascorbic acid oxidase. A similar sequence of reactions was also shown to occur by Marré & Laudi (124) using extracts from the apical parts of pea plants, and by Young & Conn (125) working with mitochondria prepared from avocado, pea seedlings and mung beans. These latter workers were able to show that the oxidation of GSH was catalysed by plant mitochondria in the presence of catalytic amounts of ascorbic acid, and that the reduction of GSSG was linked through TPN with Krebs cycle intermediates.

The biochemical evidence reviewed in this and the preceding section sug-

gests that if ascorbic acid acts as a respiratory carrier *in vivo* it is positioned between either DPN or TPN and the terminal oxidase. It emphasizes the role of DHA or other labile oxidative products as hydrogen acceptors, the concentration of which, rather than the concentration of ascorbic acid, might be expected to determine the respiratory changes through these channels.

Operation of Glutathione-ascorbic acid system in vivo.—Direct evidence that the GSH-ascorbic acid enzyme system is active in potato tissue *in vivo* is provided by the work of Barker & Mapson (126) who found that when potato tubers are placed in pure oxygen there is, after a latent period which may be as long as 50 days, first a fall in the GSH content, followed closely by a fall in ascorbic acid and by a corresponding rise in DHA. In later work (127), they found that under these conditions the fall in GSH is accompanied by a rise in GSSG, and the rate of change in the GSH/GSSG ratio is reflected in corresponding changes in that of ascorbic acid/DHA.

The maintenance of the ascorbic acid in its reduced form in this tissue thus appeared to be dependent on the maintenance of GSH. The results of these experiments support the view that the dehydroascorbic acid reductase which can be demonstrated *in vitro* in potato extracts is also operative *in vivo*. During the phase in which ascorbic acid was disappearing there was no evidence for any loss of activity of dehydroascorbic reductase; the increase of DHA, as the fall in GSH and the rise in GSSG indicate, appeared to be associated with an impairment of the system responsible for the reduction of GSSG to GSH.

An attempt to assess the importance of the GSH-ascorbic acid enzyme system as a respiratory pathway *in vivo* was made by Mapson & Moustafa (26) working with pea seedlings. In the case of the pea cotyledon it was possible to prepare extracts the respiration of which was, within experimental error, identical with the respiration of the intact cotyledon. An analysis of particulate (mitochondrial) and nonparticulate (soluble) fractions indicated that although many of the enzymes were present in both fractions, only in the soluble fraction was dehydroascorbic acid reductase present, and the concentration of glutathione reductase in the particulate fraction was only about one-fifth of its concentration in the soluble fraction. Using specific inhibitors, and determining the rate of reduction of DHA by the enzymes and substrates contained in the soluble fraction of the cytoplasm, conclusions were reached showing that about half of the respiration of the nonparticulate fraction of the cytoplasm or, about 20 to 25 per cent of the total respiration of the cotyledons, might pass over this route. Evidence was also obtained that the GSH-ascorbic acid system was functioning in the detached embryo, but owing to a lack of correspondence between the oxygen uptake of extracts and the respiration of intact tissue, no quantitative estimate of its importance could be made.

Complementary to this work was that of Young & Conn (125) who were unable to find any evidence that the reduction and oxidation of glutathione

played any large physiological role in the respiration of the mitochondria from avocado pears or pea seedlings. Thus, the addition of catalytic amounts of GSH and ascorbic acid to particulate preparations which were actively oxidising succinic or citric acids by means of cytochrome oxidase did not result in an increased oxygen uptake. This is perhaps not surprising in a tissue fraction containing a very active cytochrome system. Mapson & Moustafa pointed out the possibility of competition between different electron transferring systems for the electrons passing over a common coenzyme. In such circumstances the relative affinities of enzymes such as TPN-cytochrome-*c* reductase and glutathione reductase would presumably determine the extent of the electron transfer over either system, assuming the enzymes to be present in a homogeneous solution.

It seems appropriate to refer at this stage to the work of Stern & Timonen (27), which raises the possibility that the GSH-ascorbic acid system may play some part in the respiration of nuclei. These authors, working with plant and animal cell nuclei, found a substantial concentration of GSH and ascorbic acid in the nucleus but, in agreement with Beinert (128), no or very little cytochrome pigment. Glutathione reductase was present in the nuclei to the extent of 20 per cent of the total content of the cell. They also showed that the ascorbic acid concentration in the nuclei of the lily anther doubled during nuclear division.

The evidence reviewed here indicates that we cannot exclude the possibility that the GSH-ascorbic acid system may function as a respiratory pathway for some of the components of the cell. It seems clear, however, that although the proportion of the total respiration that passes over this route is small, it may nevertheless be of importance to those cellular components with which it is associated. It seems equally probable that the enzyme system associated with the electron transfer between DPNH and a labile oxidation product of ascorbic acid may function to some extent *in vivo* though to date we have no estimate of its importance.

Has the GSH-ascorbic acid system a function other than that of respiratory mechanism? Its role in maintaining both glutathione and ascorbic acid in the reduced state is obvious, and in this connection DHA may act as a competitor with other systems in which GSH participates, in particular with the maintenance of sulphydryl enzymes in an active state. During the very early stages of germination in the pea when respiratory enzymes are being mobilized, the full GSH-ascorbic acid system is inoperative owing to the absence of an oxidase capable of oxidising ascorbic acid [Mapson & Moustafa (26)].

The results of Marré and co-workers (129, 130, 131) are also of interest in this connection for they have reported that DHA at a concentration of 5×10^{-4} *M* strongly inhibits succinic dehydrogenase and glucose-6-phosphate dehydrogenase present in mitochondrial preparations from pea stems; and Bernheim *et al.* (132) have reported inhibitions of several oxidases in liver mitochondria by ascorbic acid. It would be useful to ascertain whether these

inhibitions are due primarily to oxidation products of ascorbic acid, or to a shift in the ratio of the GSH/GSSG level in the cell.

Ascorbic Acid and Phosphorylation

Oxidative phosphorylation.—Judah (133) was the first to observe an esterification of inorganic P coupled with the oxidation of ascorbate, and concluded from the oxygen consumed that the oxidation of ascorbic acid but not of DHA was responsible for the phosphorylation observed. Lehninger (134) studied the oxidation of a number of reducing substances by liver mitochondria in presence of cytochrome-*c* and P^{32}. The reducing agents reduced cytochrome-*c* which was then oxidised by cytochrome oxidase, but the only reducing agent to give rise to the incorporation of P^{32} into ATP was ascorbate.

The finding that a labile intermediate may be formed during the oxidation of ascorbate raised the possibility that such a substance formed by oxidation with ferricytochrome-*c* might act as a donor to the respiratory chain thereby yielding phosphorylation associated with the reduction of cytochrome-*c* rather than with its oxidation. This explanation was rendered unlikely by the observation that antimycin in concentrations which blocked the respiratory chain between substrate and cytochrome-*c* had only a slight effect on the phosphorylation with ascorbate [Maley & Lardy (135); Lehninger (134)]. Nielsen & Lehninger (136) and Slater (137) showed that phosphorylation was associated with the direct oxidation of ferrocytochrome-*c* in the absence of ascorbate, the P:$\frac{1}{2}$ cytochrome-*c* ratios being about the same as the P:O ratios obtained with ascorbate and catalytic amounts of cytochrome-*c*. Maley & Lardy (135) have finally shown that 3,4 dihydroxyphenylalanine or adrenalin can replace ascorbate in the system. It appears to be established, therefore, that the phosphorylation observed is not associated with the oxidation of ascorbic acid but rather with the oxidation of cytochrome-*c*.

Nor is there any evidence to suggest that oxidative phosphorylation occurs when electrons are transferred via the GSH-ascorbic acid system. Young & Conn (125) could find no evidence of the esterification of inorganic P either with GSSG as electron acceptor during the oxidation of Krebs cycle intermediates by avocado mitochondria, or during the aerobic oxidation of GSH in the presence of catalytic amounts of ascorbic acid. These last observations would indicate that particulate GSSG reductase and ascorbic acid oxidase do not function in any phosphorylation process, although, as the authors point out, the destruction of labile enzyme systems during the preparation and isolation of mitochondria cannot be ruled out.

Photosynthetic phosphorylation.—Arnon and co-workers (138) have shown that isolated spinach chloroplasts under the influence of light required the presence of ascorbic acid, Mg^{++}, FMN, and vitamin K in order to esterify inorganic P into the pyrophosphate bonds of ATP. No molecular oxygen was evolved or consumed during this reaction and the whole process pro-

ceeded more efficiently under anaerobic than under aerobic conditions. Although the concentration of ascorbate required to obtain the optimum effect was relatively large, the vitamin was not utilised during the reaction, and thus appeared to be acting catalytically. It seems probable that while Mg^{++} has a catalytic function in the transfer of phosphate groups, ascorbic acid, FMN, and vitamin K are acting as electron carriers.

These results of the Berkeley group on the need for ascorbic acid for photosynthetic phosphorylation have been confirmed by Whatley *et al.* (139). Computations based on known redox potentials support the conclusion that FMN is the primary electron acceptor, with ascorbic acid positioned between this constituent and the ultimate electron acceptor, the oxidised product of the photolysis of water.

Ascorbic Acid as a Hydroxylating Agent

It has been known for some years that in scorbutic animals there is an abnormality in tyrosine metabolism [Sealock & Silberstein (140)]. On administration of L-tyrosine to scorbutic guinea pigs, *p*-hydroxyphenyl pyruvic and *p*-hydroxyphenyl lactic acids were recovered from the urine, but these compounds were not seen if L-ascorbic acid was given simultaneously with the tyrosine. Similar observations were made in infants (141). This work was followed by the work of Lan & Sealock (142), Sealock *et al.* (143) and Painter & Zilva (144) in which the oxidation of L-tyrosine was shown to be catalysed by ascorbic acid in enzyme preparations from liver tissue. In view of the similar effects of D-arabo-ascorbic acid, D-gluco-ascorbic acid, and reductone, a coenzyme function of ascorbic acid in tyrosine metabolism was proposed with the enediol structure as a specific requirement. Later workers [La Du (145)], however, found that hydroquinone could replace ascorbic acid *in vitro* but not *in vivo*.

Model systems consisting of ascorbic acid, iron and oxygen have been found to catalyse the hydroxylation of many aromatic compounds [Udenfriend *et al.* (146); Dalgliesh (147)]. The hydroxyl group is introduced with the aromatic nucleus at an electronegative site. Anthranilic acid, kynurenine, tryptophan and tyrosine were all hydroxylated in positions *ortho* or *para* to the N atom attached to the ring. Phenylalanine was hydroxylated in positions both *ortho* and *para* to the side chain, and the monophenols so produced were further hydroxylated in positions *ortho* and *para* to the hydroxyl group. An existing hydroxyl group determines the orientation on further substitution; finally the side chain was eliminated with the production of diphenols, e.g., hydroquinone.

These oxidative properties of ascorbic acid had been reported by earlier workers (148, 149, 150) who considered that H_2O_2 formed during the metal catalysed oxidation of the vitamin was the agent responsible. Udenfriend and his co-workers found, however, that (*a*) H_2O_2 itself did not cause hydroxylation, (*b*) ascorbic acid alone was ineffective under anaerobic conditions, and (*c*) under such conditions hydroxylation proceeded rapidly in the presence of both ascorbic acid and H_2O_2.

There has been much speculation as to the nature of the hydroxylating agent, which seems to arise during the metal catalysed oxidation of ascorbic acid. The function of the metal has been explained as being necessary to catalyse the formation of H_2O_2 and this is believed to react with a further molecule of ascorbic acid to give a reaction product which is the actual hydroxylating agent. Dalgliesh (147) has suggested this may be a free radical from monodehydroascorbic acid but it seems equally probable that a metal chelated with ascorbic acid may be the actual catalyst.

The reaction is not specific for L-ascorbic acid since other dienols D-arabo-ascorbic, dihydroxy-maleic acids or substances containing a diketo group, DHA and 2,3 dioxo-gulonic acid, were able to replace the vitamin. This low specificity does not necessarily exclude the possibility that ascorbic acid may function as a hydroxylating agent *in vivo*, and in fact both Udenfriend and Dalgliesh have emphasized that the system is of importance in that it brings about hydroxylation of the same type as that brought about by feeding aromatics to animals or by specific enzymic hydroxylating agents. It seems unlikely that the system will contribute appreciably to the metabolism of substances for which rapid specific metabolic pathways exist although Udenfriend *et al.* (146) reported that in scorbutic guinea pigs, hydroxylation of aromatics fed to the animals was reduced. The possibility of the system participating in melanin formation and in the production of many phenolic derivatives in plants is obvious.

There is at present no evidence of the existence in plants of any hydroxylating enzyme which requires ascorbic acid for its activity, but one such catalysing the hydroxylation of *p*-hydroxyphenyl-pyruvic acid has been found in animal tissue [Uchida *et al.* (151); Knox (152)]. As with the non-enzymic systems L-ascorbic acid may be replaced by D-arabo-ascorbic, D-gluco-ascorbic acid and each by substances such as 2,6 dichlorophenolindophenol. The function of ascorbic acid in this enzyme system may be connected with the oxido-reduction carrier properties of the vitamin, or to some direct effect of ascorbic acid on the enzyme. In any case, it is premature to call ascorbic acid a coenzyme for the reaction.

Ascorbic Acid and the Mobilization of Ferrous Iron

It is well known that certain enzymes require ferrous iron for their activity. This was established for aconitase by Dickman & Cloutier (153) and by Morrison (154), and for homogentisicase, the enzyme responsible for the rupture of the benzene ring of homogentisic acid, by Suda & Takeda (155), Crandall (156), and Schepartz (157). Takeda (158) showed that homogentisicase from which the metal had been removed could be restored to activity by the addition of ferritin (iron storage protein found in liver tissue) and ascorbic acid, the function of the vitamin being attributed to its ability to reduce ferritin to the ferrous state and transfer the metal to the enzyme. The Japanese workers advanced the hypothesis that a primary function of ascorbic acid is the mobilization to the cells of ferrous iron. Support for this idea was found in experiments with scorbutic guinea pigs, and with normal

animals injected with α,α'dipyridyl, in which the homogentisicase activity of the tissues was found to be lower than in tissue from normal animals. In an extension of these studies to a determination of the activity of aconitase, in normal, scorbutic and α,α' dipyridyl injected guinea pigs, they found that the decrease in the enzyme activity of liver mitochondria ran parallel with the development of the scorbutic condition, and that addition of ferrous iron and ascorbic acid *in vitro* partially restored the enzyme activity. Similar results to these were obtained with the animals injected with α,α' dipyridyl. Associated with this decrease in activity of aconitase was evidence that a disturbance had occurred in the tricarboxylic acid cycle, notably an impairment in the ability of the animal to oxidise citrate. These results, although of interest, do not suggest that this function of the vitamin is highly specific, but they do revive the idea that ascorbic acid may act, by reason of its reducing potential, to maintain the activity of different enzyme systems.

The beneficial effect of ascorbic acid on the utilization of iron salts in animals has also been demonstrated by Greenberg & Rinehart (159) and by Bagchi & Chowdhury (160), but as far as the writer is aware there is no evidence as yet to indicate whether ascorbic acid has any influence on the metabolism of iron in plants.

Action of Ascorbic Acid on Enzymes

The activities of several enzymes appear to be markedly influenced by ascorbic acid. In some cases the effect observed is one of activation, in others of inhibition. Cathepsin is activated by ascorbic acid and the effect is increased in the presence of iron salts [Euler *et al.* (161)]. Activation of arginase by ascorbic acid has been reported by Edlbacher & Leuthardt (162). Papain is inhibited by ascorbic acid alone but activated in the presence of ferrous salts [Maschmann & Helmert (163)]. There is evidence suggesting that the ascorbic acid-iron complex activates by first reducing dithiol compounds associated with the enzyme, and that these thiol compounds in turn activate the enzyme [Purr (164)]. Similar reactions may be involved in the activation of arginase by the ascorbic acid-iron complex [Purr (165)].

Many enzymes have been shown to depend for their activity on the integrity of an —SH group in the molecule [Hellerman (166)], and the effect of ascorbic acid has been suggested as being due to the protection of such —SH groups from oxidation [Harrer & King (167)]. However, it has been shown that the activity of one such enzyme, urease, is inhibited by ascorbic acid, although this inhibition was eliminated in the presence of cysteine [Elson (168)]. A suggestion that dehydroascorbic acid was the agent responsible for reacting with the —SH group of the enzyme and that the protective effect of thiol compounds was due to the reduction of dehydroascorbic acid [Quastel (169)] was found to be untenable when it was shown that dehydroascorbic acid did not inactivate urease [Giri & Seshagiri Rao (170)]. In further studies [Mapson (171)], it was found that ascorbic acid itself does

not inhibit urease activity. In the presence of Cu^{++}, however, it does so by effecting the reduction of $Cu^{++} \rightarrow Cu^{+}$, which latter ions have a much higher affinity for —SH groups than have the former. With mercury salts the reverse effect of ascorbic acid was observed, namely, an activation of urease activity. This was correlated with the fact that enzymic activity was reduced more by Hg^{++} than by Hg^{+} salts. The action of ascorbic acid on urease could, therefore, be explained in terms of its reducing action on metallic ions present in solution. The inhibiting action of ascorbic acid on plant β-amylase is probably explicable on a similar basis, since it has been shown that the inhibition was increased in the presence of small amounts of copper salts [Hanes (172)].

The action of ascorbic acid on many enzymes appears to be conditioned by the presence of metallic ions; certainly in a number of cases where its action has been critically examined, this has been found to be so. The inhibition of potato polyphenolase by ascorbic acid (173) is claimed, however, to be due to an effect of the vitamin per se on the protein moiety of the enzyme which affects the combination of the apoenzyme with Cu^{++}. Whether ascorbic acid *in vivo* has any regulating influence on these enzymes is uncertain. With xanthine oxidase which has been shown to be inhibited by traces of ascorbic acid *in vitro* (174), no evidence was obtained to show that the vitamin, even in doses several hundred times greater than the physiological requirement, had any significant effect *in vivo* (175).

Assimilation of Nitrate

Von Hausen (176) observed that if the cotyledons of pea seedlings were removed five to nine days after the start of germination and the cotyledonless seedlings transferred to a sterile nutrient solution in which nitrogen was supplied as nitrate, growth, which was at first feeble, soon ceased. If ascorbic acid in a concentration of 30 to 40 mg. per litre of nutrient solution was added, the plants grew until the flowering stage. In a continuation of the work, Virtanen & von Hausen (177) found that it was necessary to add three times this level of ascorbic acid to promote growth equal to that observed in normal plants. When similar experiments were carried out with ammonium sulphate as the source of nitrogen, in both wheat and pea seedlings, normal growth was observed in the cotyledonless seedling even in the absence of ascorbic acid. Later experiments showed that other reducing substances, e.g., glutathione, cysteine, and reductone could also promote growth under these conditions, and the authors suggested that the sulphydryl compounds act by conserving the small amounts of ascorbic acid present.

The suggestion that ascorbic acid may be essential for the assimilation and utilization of nitrate nitrogen by the plant received some support from the work of Hewitt and his associates (178). In molybdenum deficiency there is both a reduction of ascorbic acid in the tissue and an impairment in the reduction of nitrate as evidenced by the accumulation of nitrate in the leaf and by the lowered ability of homogenates of leafy tissue to reduce nitrate *in vitro*.

The isolation and characterisation of nitrate reductase as a flavo-protein with molybdenum as the metal component (179) renders intelligible Hewitt's findings of the accumulation of nitrate in molybdenum deficient plants but suggests no role for ascorbic acid in this reduction. Nor has any role been found for ascorbic acid in the reduction of nitrite to ammonia by nitrite reductase isolated from soybean leaves by Nason *et al.* (180) or on the corresponding reduction of hydroxylamine to ammonia by hydroxylamine reductase from *Neurospora crassa* (181).

Ascorbic Acid and Growth

Mention has already been made of the experiments of Reid (5) who, from a study of the concentration of ascorbic acid in cells, observed a correlation between surface area of cells and their concentration of ascorbic acid. Conflicting reports of the effect of ascorbic acid on growth of young seedlings have appeared. Kögl & Haagen-Smit (182) were unable to detect any effect of added ascorbic acid on the growth of young pea seedlings, but Havas (183), Davies *et al.* (184), and Dennison (185) found that ascorbic acid promoted the growth of certain plants. Bonner & Bonner (186) observed a stimulative effect on growth by adding the vitamin to cultures of excised embryos of certain varieties of plants but negative effects with others. Correlated with these observations were some which showed that those varieties giving negative results were able to synthesize the vitamin to a much greater extent than those in which positive effects were observed. The fact that some varieties did not respond to feeding with ascorbic acid with an increase in growth may simply mean that they are able to synthesize sufficient of the substance for their needs; consequently, negative results do not necessarily indicate that the substance is not a growth factor.

Raadts & Söding (187) found that dehydroascorbic acid stimulates the growth of *Avena* coleptiles. They suggest that this was due to the greater formation of indoleacetic acid from some precursor. However, they also observed that methylene blue and, in some cases, hydrogen peroxide had similar effects. Wetmore & Morel (188) let auxin diffuse from *Equisetum* tissue to agar blocks and found that the addition of ascorbic acid to the receiving blocks greatly increased the curvatures obtained in the *Avena* test.

Tonzig & Trezzi (189), on the other hand, suggest that ascorbic acid prevents the formation of an indoleacetic acid protein complex which is the actual growth stimulant. Clearly there is little to suggest that ascorbic acid has any specific growth stimulating effect other than that of being a necessary component of growing cells.

Conclusion

We have summarised in this review the known characteristics of ascorbic acid and attempted to indicate to what extent these may represent its function *in vivo*. In animals it seems compelling to regard the vitamin as specifically essential for some precise metabolic step. Thus, the scorbutic con-

dition of an animal cannot be relieved by any nonspecific reducing agent, and such a specific role should be referable to participation in one or more enzyme systems which can be demonstrated biochemically. Since no plant has as yet been rendered scorbutic we have no conclusive evidence that ascorbic acid is similarly essential to the plant cell. On the other hand, the universal occurrence and localization of the vitamin in the most active metabolizing cells suggests that it has an essential function in plants as in animals. It seems highly improbable that, in view of the alternative respiratory mechanisms now known to exist in plants, its function is solely that of an electron carrier. The present state of our knowledge is a challenge to enzymologists to determine the proper place of this substance in the metabolism of the plant cell.

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PHYSIOLOGY OF THE TOBACCO PLANT[1,2]

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Introduction

The tobacco plant (*Nicotiana tabacum* Linnaeus) has served as a useful subject in pioneering investigations of special interest to the plant physiologist. These include virus studies, daylength, mineral deficiency, organic acids, alkaloids, nitrogen metabolism, and inanition (curing).

Tobacco is a leaf crop utilized for its physiological action, mainly through combustion. Its effects are due largely to the presence of an alkaloid, nicotine. Inhalation of the fumes of combustion can be disagreeable unless proper treatment of the harvested leaf—"curing," "aging," and "fermentation"—have resulted in destruction of irritating ingredients and development of a pleasing taste and aroma. Ability of the leaf to glow without flame when ignited is an important property and referred to as "fire-holding capacity," i.e., duration of glow in seconds. Specially developed varieties are employed for the different types of tobacco—flue-cured, burley, Maryland, cigar, etc.—that may be blended in the process of manufacture. *Nicotiana rustica* Linnaeus, the tobacco of pre-Colonial days in North America, is also grown abroad in some quantity. None of the 58 other *Nicotiana* species, some employed likewise by natives, are grown as crops according to Goodspeed (68), in his book on the genus *Nicotiana*.

A special terminology has developed with relation to field operations that differs to some extent with locality. Seed is sown and the seedlings grown in outdoor seedbeds before setting in the field. Decapitation of the plant at about time of blossoming is referred to as "topping," and removal of branches that subsequently develop through loss of apical dominance is called "suckering." The green leaves are excised or "primed" as they mature, or the entire top is removed and hung in the barn to "cure." A roughly regulated regime of ventilation, temperature and humidity for the slowly drying leaves favors those catabolic processes considered desirable.

Garner (60) states commercial tobaccos require about 70 to 110 days to mature after setting in the field. The time needed to produce suitable

[1] The survey of literature pertaining to this review was concluded in June, 1957.

[2] A joint publication of the Agricultural Research Service, U.S.D.A. and the Maryland Agricultural Experiment Station (Contribution 2815, Scientific Article A 633).

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seedlings (5 to 6 in.) in outdoor seedbeds is about 40 to 50 days after germination. The leaves of tobacco cured on the stalk are all harvested at the same time but harvesting may last 6 weeks when the leaves are removed individually (flue-cured). Drying of the crop takes about 3 weeks for stalk-cured tobacco and 4 days for flue-cured.

The physiology of the tobacco plant was reviewed briefly by Garner in in his book on the production of tobacco (60) published in 1951, which should be consulted for additional information concerning varieties and other details of production. Therefore, this review covers, in the main, reports appearing subsequent to this date. It deals primarily with the physiology, nutrition, and metabolism of the tobacco plant and the immediate post-harvest changes in the leaf. Selection of topics has been made, however, with cultural procedures in mind in order to emphasize the special problems encountered by the physiologist in tobacco culture. No claim is made for completeness in coverage.

Seed, Seedbeds, and Transplants

Seed.—Tobacco seed, as that of other plants, deteriorates after long storage. Seed of certain varieties requires a light stimulus for germination. Pyriki & Kunstmann (148) found germination capacity decreases to be accompanied generally by increases in per cent of free fatty acids. Citric acid decreased under the same conditions. Age of seed affected embryo viability and rate of growth (35). Red and ultraviolet radiations were most effective in breaking dormancy of light-sensitive seed, while high temperature and lipide extraction were only partially effective [Gimesi *et al.* (64)]. The light intensity required to overcome dormancy varied inversely to length of soaking [Isikawa (85)]. Intermittent light was more effective than continuous light in breaking dormancy according to Kerr (92).

Seeds accumulate selectively magnesium, phosphorus, and nitrogen and exclude calcium, sodium, chlorine, and silicon salts [Cooper *et al.* (37)]. Treatment of seed with β-indoleacetic acid, 2,4-D, and hexachlorocyclohexane gave no improvement in growth [Hitiér & Izard (79)]. Tobacco seed was very sensitive to injury by 2,4–D [Giraud (66)] and by maleic hydrazide [Nétien & Briffaz (131)].

Seedbeds.—No record was found in the recent literature concerning possible correlations between rate of seeding of the seedbeds, type of seedling growth, and yields in the field. According to Steinberg (173), however, premature blossoming of seedlings after transfer to the field could be due to low seedbed temperatures and use of overage seedlings. Volodarsky (223) noted that an initial deficiency of nitrogen followed by an increase in supply resulted in a considerable decrease in the time required for flowering.

Prevention of competing weed growth in the seedbed was originally based on use of high temperature for destroying the weed seed in the soil, i.e., by burning of brush, and later by the use of steam. Pathogenic organ-

isms were also thereby decreased or eliminated. The efficiency of a newer method employing large quantities of cyanamid plus urea was studied by Volk (221). More recently, still another method has been actively studied, by Stover & Koch (187), and by Thomson & McLeod (189), i.e., the use of methyl bromide. Its advantages consist, apparently, in its effectiveness against weeds serving as hosts for tobacco mosaic virus, its lower residual toxicity, and its increased effectiveness in heavy soils and at higher latitudes. Hill, Klingman & Woltz (77) should be consulted for additional details.

Transplantation.—Planting in the field after pulling from the seedbed is a shock to seedlings, even though done in moist soil. Growth is not resumed for a couple of weeks even if the seedling survives. Root-forming action of synthetic hormones has been found to be of no value by Liquori & Martino (106); the use of starter solutions for bettering survival or hastening root formation is also ineffective [Hutcheson & Woltz (83]. The use of greenhouse-grown seedlings has been recommended on the ground of greater uniformity in stand of plants by Raeber (149). A study of nitrogen and phosphorus nutrition led Muraoka & Tomoyoshii (127) to conclude that rooting capacity of seedlings was associated with the carbohydrate content and size of the seedling. Also, the higher the dry weight of the transplanted seedling, the earlier it flowered.

Responses to Physical Environment

Information on the influence of physical environment on the tobacco plant continues to accumulate slowly. Tobacco, of course, is a day-neutral plant except for certain Mammoth varieties. Evidence presented here (see seedbeds also) illustrates, nevertheless, the very marked effects of light, temperature, and moisture on growth, flowering and metabolism of the tobacco plant.

Light.—Maryland Mammoth tobacco, a short-day plant whose study led to the discovery of photoperiodism by Garner and Allard, can flower on long daylengths when subjected to gamma radiation [Vlitos, Shapiro & Meudt (220)]. Nicotine increased with hours of sunshine, decreased rainfall, and increased temperature [Renier (152)]. The photosynthetic path in tobacco with $C^{14}O_2$ was found by Doman *et al.* (48) to vary from that in other plants [see also Nezgovorova (132)]. Quality of light may affect quantitative results with $C^{14}O_2$ according to Voskresenskaya (224). Increases in light intensity caused a rise in extent of phototaxis of chloroplasts and increased the spread between upper and lower thresholds of response [Babushkin (13)].

Temperature.—The importance of low temperature, particularly at night, in the physiology of the semitropical tobacco plant is receiving increased emphasis. Camus, Eggman & Wildman (32) found night temperature profoundly affected total amounts and proportions of cell walls, particulate proteins, and soluble cytoplasmic proteins. Growth habit and rate,

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time of flowering, and final weights were also markedly affected by night temperature in studies by Camus & Went (33). Low night temperatures hastened flowering and decreased leaf number according to Saito (154). Continuous high temperature and humidity each retarded blossoming [Coolhaas (36a)]. High temperatures and long daylengths inhibited flowering according to Muraoka & Ohori (126a). Flowering was most rapid with an 8 to 10 hr. day at 13 to 18°C. A low temperature regime before transplanting hastened subsequent maturity [Steinberg (173)]. Thermoperiodic effects varied with variety [Camus & Went (34)]; Long & Porizo-Giovanola (107)]. Mammoth Rustica tobacco flowered with night temperatures of 50 to 60°F., irrespective of daylength [Steinberg (174)]. Temperatures over 25°C. increased the inhibiting effect of the root tip on formation of side-roots, the inhibiting effect disappearing on excision of the apical meristem [Geissbühler (61)].

Moisture.—Comparative tests indicated that some 10.5 to 18.5 per cent of soil water saturation capacity was unavailable for most tobacco varieties, though one variety was found for which inactive moisture was only 4.1 per cent of saturation. Resin content was maximum with 41 per cent soil moisture content, while carbohydrate and protein decreased with high soil moisture (6). Growing shoots are considered by Jackson (86) to be dependent on functional root systems for growth factors other than water and minerals. Tobacco is very susceptible to flooding according to Kramer (94, 95). Kramer & Jackson (96) found that similar symptoms of injury could be produced by substituting CO_2 or N_2 for the soil atmosphere. Freezing-point determinations of osmotic pressures of expressed sap varied with variety of tobacco and also, in *Nicotiana,* with species [Puzzilli (144, 145)]. Decrease in available soil moisture led to increased oxidase, catalase, and ascorbic acid of the leaf and glutathione decreased [Tombesi (193, 194)]. Irrigation depressed total nitrogen and nicotine and increased potassium and fire-holding capacity of leaf in trials by McMurtrey *et al.* (112). The beneficial effects of soil irrigation under normal conditions of crop production have been emphasized in great detail by van Bavel (204) and Westbrook (228).

Mineral Nutrition

As with other plants, the bases of interpretation in mineral nutrition studies of the tobacco plant often differ. Carbon level (total photosynthate minus respiration) can greatly influence results. The factors of limited growth capacity, nutrition level, mineral balance, presence of harmful substances, and effects of physical environment may sometimes be overlooked in planning and interpretation of experiments. These are factors in the growth of all organisms. In this review each essential element discussed is considered a priori to function in the total life of the plant, instead of being accepted as indispensable for single physiological processes as in the past. It should also be kept in mind that field studies with flue-cured and

Maryland type tobaccos are based on restricted nitrogen supply, and therefore limited growth.

Macronutrients.—McEvoy (109) noted low nitrogen accelerated maturity and decreased content of other macronutrients, except phosphorus, in the leaf. When the ratio of ammonium to nitrate nitrogen was high there was an increase in insoluble and soluble nitrogen, amide, and alkaloid in experiments by Gilmore (63). Similar results were obtained by Vladimirov & Lapshina (217), who also noted that maximum storage capacity of the plant for nitrate salts was several times higher than for ammonium salts under these conditions. Stale & Bovay (163) state that form of nitrogen was without influence on nitrogen content of the leaf. Mothes & Trefftz (126) found spraying with 0.2 *M* NH_4NO_3 can take care of the full needs of the plant for nitrogen. Delwiche (43) found nitrate-N^{15} was readily converted to amino-, amide-, and ammonium-N in light or dark in excised leaves, and ammonium-N was not oxidized to nitrate-N. Aseptic-culture tests with reduction products of nitrate-N revealed nitrohydroxylaminic acid ($H_2N_2O_3$) was readily available as a sole source of nitrogen, nitrite-N ($H_2N_2O_4$) was a poor source, and hyponitrite-N ($H_2N_2O_2$) and hydroxylamine were very poor sources [Steinberg (176)]. Leaf absorption of N^{15}-urea was increased tenfold by breaking of epidermal hairs [Volk & McAuliffe (222)].

Delayed maturity resulted from low phosphorus, leading to decreased nitrogen and magnesium content of the leaf [McEvoy (109)], and leaf abscission [Komatsu (93)]. Komatsu (93) also found high phosphorus delayed maturity, increased the nitrogen content, and delayed attainment of a peak in soluble carbohydrates. In addition, middle leaves, before topping, contained more pectin, crude cellulose, lignin, malic acid, and total resin; and less protein, nicotine, oxalic acid, wax, and gum. From 15 to 68 per cent of phosphorus added to the soil could be recovered in the plant by Woltz, Hall & Colwell (235). Radioactive phosphorus as KH_2PO_4 was transported from root to apex at the rate of about one inch per 1.4 minutes [Weber (227)]. Tissue-bound acid labile P seems to be in the form of pyrophosphate combined with protein [Wildman, Campbell & Bonner (230)].

High potassium led to brighter cured leaf color [Tumminello (203)], and increased thiamine synthesis [Ovcharov (134)], while lactic acid decreased. Seed yield was increased by high potassium [Perucci (137)].

Blume & Hall (17) studied absorption of radioactive calcium from soil. Calcium and magnesium optima for growth were unaltered by initial nutrient solution acidities (pH 4 to 7) [Steinberg (170)]. High $(NH_4)_2SO_4$ induced Mg-deficiency symptoms [McEvoy (110)]. Ratios of chlorophyll to xanthophyll to carotene were not appreciably disturbed by magnesium deficiency [Hinkle & Eisenmenger (78)]. High sulfur level decreased fire-holding capacity of leaf [Neas (130)]. Sodium could replace, partially, the need for potassium by tobacco [Verona (205)].

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Micronutrients.—Micronutrient studies with the tobacco plant have been reviewed recently by McMurtrey (111). A mechanism for symptom production with mineral deficiency has been suggested to consist in the accumulation of toxic quantities of free amino acids [Steinberg, Bowling & McMurtrey (180)]. Paper chromatographic determinations have been made on free amino acid content in leaf and root with deficiency in micronutrients [Steinberg (179)].

Difficulty in maintaining adequate iron nutrition in water-culture is well known. Use of chelates has been suggested, though these may possibly lead to departures from normal metabolism. An adequate level of iron even from precipitated and colloidal iron can be assured by semiweekly stirring to cover the newly-emergent root hairs of the plant [Steinberg (170)]. Ultraphagocytosis [Gosselin (69)], or the direct intake of colloid particles of 6 to 9 mμ, may be a factor in these results. Jenny's work has emphasized this possibility (89). Root tips and root-hair zones were most active in surface exchange properties [Williams & Coleman (231)]. Micronutrient deficiencies [Steinberg, Specht & Roller (184)] caused only slight changes in mineral composition of the plant as a whole but altered distribution in the plant. Ash was increased by each deficiency. These results were obtained in an optimum or "economical solution" (Hoagland), i.e., a minimal salt solution for maximum yield in a specific environment. Average absorption of minerals reached 94 per cent, and could probably be bettered. Initial mineral composition of the nutrient solution and that of the plant as a whole at flowering were practically identical.

Brown & Steinberg (25) noted that micronutrient deficiencies could cause detectible decreases in enzymes prior to onset of visual symptoms. Enzyme responses differed with and were characteristic of each type of deficiency. Similar estimates of enzyme content of the plant with age and environment would appear desirable. Manganese deficiency decreased ascorbic acid content [Maton (116)], but low boron and molybdenum were most effective in decreasing ascorbic acid [Steinberg (170)]. Tobacco requires molybdenum according to Steinberg (175) and Stout & Johnson (186). No significant differences could be detected when boron was supplied as B^{10} or B^{11} [McMurtrey & Engle (113)]. Boron deficiency, alone of the trace elements, affected alkaloid synthesis and led to marked increases in alkaloid content in association with excessive root branching [Steinberg (177); Steinberg & Jeffrey (182)]. Additional studies have appeared on both the beneficial and harmful effects of chloride ion on tobacco (51).

Pruning of Tobacco

Decapitation (topping) at onset of flowering and subsequent debranching (suckering) are standard practices in production of tobacco (60). The effects include improved quality (60) and increased size and weight of leaf having a higher alkaloid content [Woltz (234)]. Osmotic pressure is

unaffected [Puzzilli (146)], likewise protein content [Anitia & Dimofte (7)], but soluble sugar increases. It has been demonstrated that manual topping and suckering lead to an acceleration in root branching and weight of roots that would seem to have a causal relation to increased alkaloid synthesis [Steinberg & Jeffrey (183)]. Gains in leaf yield would seem to depend on presence of residual nitrogen and moisture, and may be less than those in root weight when residual nitrogen is lacking (7).

More recently, attempts to bypass manual suckering began with the use of synthetic hormones [Calvert (31); Hardesty, Scofield & Anderson (75); Steinberg (168)] to replace loss of apical dominance in the plant after topping. The use of oil or oil emulsions to kill axillary buds was suggested at about the same time (1, 31). Major attention is now focused on use of maleic hydrazide to prevent development of the axillary buds after topping [Petersen (138); Petersen & Naylor (139)]. Variations in reported results indicate that each of the three methods under investigation is sensitive to environmental factors which modify responses. Synthetic hormones and maleic hydrazide, moreover, may cause leaf abnormalities (139, 168). Use of oil may result in bacterial stem rot and loss of leaves (1). The objective in chemical suckering of tobacco is a compound that would eliminate the necessity of both manual topping and suckering without harmful alterations in the leaf.

Petersen & Naylor (139) have reported that maleic hydrazide treatment may cause marked changes in composition of tobacco leaf. These changes include increases in free amino acids, asparagine, glutamine, ammonia, reducing sugar, and calcium. Protein and phosphorus decreased. Arnaud (8) found an increase in both starch and soluble sugar. Flowering is inhibited [Naylor (129)]. Yield, quality, and burn appeared unaffected [Petersen (138)]. Chloroplasts were larger but fewer in number after maleic hydrazide treatment of the plant, and respiration increased [Callaghan & Van Norman (30)]. Rate of absorption of maleic hydrazide has also been determined by Zukel *et al.* (240).

Frenching

A noninfectious physiological disorder of tobacco, known as "frenching," has been of great interest to growers since Colonial times (60). It usually appears as a network chlorosis of apical leaves, followed by formation of progressively narrower leaves. In extreme cases there is no expansion of lamina in younger leaves (strapping) or stalk elongation. Its appearance and disappearance are erratic. The tomato plant and some of the other *Nicotiana* species are also stated to be susceptible.

Frenching has been attributed to many different causes. Simulation of the symptoms of frenching may also be caused by many compounds including 2,4-D, naphthylacetic acid, and indoleacetic acid [Steinberg (164)], and maleic hydrazide [Middelburg (121)]. In no case are symptoms identi-

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cal with those of frenching, though they might be an indication that inhibition of laminar growth with some toxic compounds takes place at a lower level than does that of midrib growth.

Recent investigations by Steinberg (166, 169, 172) make it seem probable that frenching is caused by an organic toxin produced by *Bacillus cereus* Frankland and Frankland. Increased populations of the bacillus have been found in adjacent soil and rhizospheres of frenched plants (171). Diffusion products of these bacteria and the dead bacteria cause symptoms of frenching in aseptically grown tobacco seedlings (166, 169). The nature of the toxin has not been demonstrated as yet.

Free L-isoleucine, however, causes frenching in aseptic culture (167). Its capacity in this respect is exceeded fourfold by L-alloisoleucine (169). Four p.p.m. of the latter produces typical frenching. The dextro-forms produce other symptoms of toxicity. Leaves of frenched plants are high in L-isoleucine (181). L-alloisoleucine is a component of actomycin C (23).

Frenching has been produced in the greenhouse (91) but low nitrogen, liming to neutrality, and use of excess dicalcium phosphate were necessary with normal soils (178). Production of frenching capacity in the beds was paralleled by high *B. cereus* counts. High nitrogen could be used to mask the symptoms of this disorder in newly formed tissue but reversion to symptom formation took place when nitrogen again became depleted.

The results enumerated illustrate the difficulties that can sometimes be encountered by ignoring the soil microflora in physiological studies. Here a metabolic product of a soil bacillus having a formative action on the plant would seem to be the primary causal factor. While no free amino acids have been found to be present in soil (22, 172), there would seem to be no doubt that they can be absorbed when present (62, 218). The low concentrations of L-alloisoleucine, if this be the effective toxin, of about 2 to 5 p.p.m. would be difficult to determine, particularly if, as seems to be the case, the bacteria are largely localized on the surface of the roots.

Organic Metabolism[3]

Little attention seems to have been taken of a gradual but profound alteration in a former concept of the physiologist. Compounds formerly excluded *a priori* from consideration in metabolism as "poisons" are now accepted as normal metabolites. This change in viewpoint is based on studies at very high dilutions, which the greatly increased sensitivity of present analytical methods makes possible. Even slight abnormal accumulations of such highly reactive compounds would be expected to lead to marked changes in metabolism and morphology of the plant.

A degree of caution might be desirable in interpreting data based on plant macerates. Mutual interactions of hitherto localized cell components

[3] Unless otherwise stated, what follows deals with the green leaf of the tobacco plant.

may seriously affect rates of enzymatic reaction, or even make possible enzymatic reactions not normally present. A comparison between rates of reaction *in vivo* and *in vitro* might, moreover, indicate relative degree of importance in normal metabolism.

Carbohydrates.—Paper chromatographic tests revealed the presence of raffinose, sucrose, glucose, fructose, and an unidentified sugar with 3-4 C atoms in seed [Scarascia, Venezian & Giovannozzi-Sermanni (156)]. Raffinose disappeared after germination, while maltose and ribose appeared. All sugars decreased at the transplant stage, but sucrose, glucose, fructose, and ribose were always present in adult plants. Vittorio and co-workers (215), using C^{14}-labeled glucose and glucose-1-phosphate for excised leaf culture, found that the fructose component of sucrose is derived from the glucose and not from the free fructose present in the leaves. There was a preferential transformation of the glucose supplied into sucrose and not into starch. The sugars which were fed served as respiratory substrate only in darkness. Externally-supplied sugars in aseptic culture are used with the exception of galactose [Farkas (52); Hildebrandt & Riker (76); Steinberg (165)].

Carboxyl-C^{14} of acetate entered glucose as photosynthetic CO_2 and, also, directly into the 2, 5 positions. Carboxyl carbon of formate and lactate entered glucose via the 3, 4 positions in the usual photosynthetic sequence [Krotkov, Vittorio & Reed (99)].

In tobacco leaves, C^{14} from $C^{14}O_2$ appears in a polysaccharide before it does in sucrose, glucose, or fructose. The activity also appeared earlier in sucrose than it did in fructose and glucose. It was suggested that the free glucose and fructose arose by sucrose hydrolysis [Vittorio, Krotkov & Reed (216)]. Porter & May (142) stated that fructose and glucose, whether free or combined in sucrose, were equally available for starch synthesis and CO_2 production. They suggested that these materials arose from a common pool in which hexose derivatives are rapidly equilibrated, i.e., free hexose does not participate directly in sucrose or starch synthesis.

Four C^{14} compounds with R_f's lower than glucose, after isolation from radioactive starch, were better starch formers than glucose, glucose-1-phosphate, or maltose [Vittorio *et al.* (214)]. Krotkov & Rizvi (98) found that either starch breakdown did not proceed as far as maltose or the maltose formed was utilized before its release into the metabolic pool. The main carbohydrate found by feeding maltose-U-C^{14} was sucrose, with only a small amount of starch.

Porter & Martin (141) prepared radioactive starch, glucose, and fructose from tobacco leaves after assimilation of $C^{14}O_2$. Only small amounts of C^{14} were incorporated in the leaf protein and in the cellulose, hemicellulose, and polyuronides. Skoog & Robinson (161) indicated that auxin may have a catalytic function in carbohydrate metabolism. The distribution of C^{14}-labeled sucrose was observed to be affected by the boron

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content of the plant [Sisler, Dugger & Gauch (160)]. Labelled glycine-1-C^{14}, serine-3-C^{14} and formaldehyde-C^{14} were incorporated into lignin in tobacco plant metabolism [Hamill, Byerrum & Ball (74)].

Organic acids.—The extensive studies by Vickery and his associates on the metabolism of organic acids indicated their interconvertibility in the tobacco leaf. The metabolism of malic acid [Pucher & Vickery (143)], glycolic acid, or succinic acid [Vickery & Palmer (213)], fumaric acid (212), and D-isocitric acid [Vickery & Abrahams (209] to citric acid was demonstrated. Acetic acid is also metabolized. It has been found that malic acid can be formed by oxidation of citric acid [Vickery, Hargreaves & Nolan (211)]. Oxalic acid is an end product of oxidation [Vickery & Abrahams (210)]. Stutz & Burris (188) consider malic acid a key compound in organic acid synthesis.

Zbinovsky & Burris (237) determined by the use of C^{14} acids that formic acid was converted to malic acid and acetic acid, malic acid to citric acid, and citric was converted slowly to other acids. Oxalic acid was inert. The ratio of citric to malic acid, formed from glycolic acid, was higher in the dark than in the light.

Organic acids can serve as respiratory materials for plants [Menschikova (117)]. Vickery (207) noted that when the culture solution is supplied at a reaction more alkaline that the cell solution, loss of acid components by respiration is minimized and the conversion of malic acid to citric acid is stimulated. The presence of D-glyceric acid was reported by Palmer (135). He found also that quinic acid is a normal component of the tobacco leaf and does not arise from chlorogenic acid [Palmer (136].

Ribonucleic acid [Sisakyan & Odintsova (159)] was found to be maximum in young tissues and declines with age. Asku (12) reported that the content of malic acid diminishes from the bottom leaf to the uppermost one (9.23 per cent to 0.17 per cent), whereas the reverse applies for the content of oxalic acid (2.2 per cent to 5.2 per cent). Reddi (151) stated that the ribonucleic acid of tobacco leaves is characterized by a low uracil and a high guanine content.

Protein and amino acids.—Lysine, ornithine, asparagine, histidine, arginine, aspartic acid, glycine, alanine, tyrosine, methionine, tryptophan, phenylalanine, and two unknowns were reported as products of enzymatic hydrolysis of seed extracts [Giovannozzi-Sermanni & Scarascia-Venezion (65)]. Michl & Kuhn (118) found appreciable amounts of free α-alanine, γ-amino butyric acid, aspartic acid, and glutamic acid in all types of tobacco leaf. The amount of other amino acids differed between types. Frankenburg & Zacharius (58) found most of the known plant amino acids in extracts of two types of green cigar tobacco. Aspartic acid, glutamic acid, glycine, lysine, and the leucines amounted to 65 per cent of the amino acids present in the leaf protein. The amino acids liberated through proteolysis during curing did not re-appear in their entirety in the free form, but were largely

transformed into aspartic acid and asparagine, to the extent of 85 per cent of the total. Ranjan & Laloraya (150) claim some amino acids, such as asparagine, phenylalanine, valine, and methionine, not originally present, appeared after leaf excision. A better knowledge of the free amino acid spectra of tobacco varieties and strains of pathogens will, it is believed, afford a basis for interpretation of some aspects of susceptibility and resistance to disease. Amino acids vary widely both as nutritive substrates and as inhibitors with different organisms.

Protein-N usually decreases at night [DeBonis (42)]. Due largely to deamination, relative N content diminishes with ripening and curing of the leaves (19). The release of amino acids from the roots of senescent plants has been reported [Frank (54)].

Evidence was obtained by Stephenson and co-workers (185) for a coupling of photosynthetic energy to protein synthesis under cell-free conditions. Protein N^{15} was formed in plastids and cytoplasm; in the former only with light and CO_2 [Andreeva & Plyshevakaya (4)]. Light was necessary for formation of radioactive protein with $(N^{15}H_4)_2SO_4$ and $C^{14}O_2$. The activity resided in alanine, serine, and glycine after hydrolysis of the protein [Andreeva (2)]. Andreeva (3) concluded, therefore, that protein is formed from the early intermediates of photosynthesis, and only in the dark are carbohydrates utilized as sources of energy and carbon.

Similar leaf-protein spectra were found by Singer *et al.* (158) in different species, and a single protein constituted 23 to 50 per cent of total cytoplasmic proteins. This water-soluble nucleoprotein contained ribonucleic acid in somewhat varying content, but appeared relatively homogeneous with respect to molecular weight (375,000) and electrophoretic mobility [Eggman, Singer & Wildman (49)]. High molecular weight, free ribonucleic acids were isolated from homogenates of tobacco leaf by ultracentrifugation followed by electrophoresis. Four (or more) types, more mobile than nucleoproteins, were present according to Lindner, Kirkpatrick & Weeks (105).

About 30 per cent of the phosphorus in the young tobacco leaf is present as ribonucleic acid and 7 per cent as deoxyribonucleic acid. About 70 per cent of leaf ribonucleic activity was found in the sap, of which about one-tenth was associated with the microsome fraction [Holden & Pirie (80)].

Alkaloids.—An excellent review of alkaloid physiology was recently published by Mothes (123). It should be consulted for material not summarized here. Nicotine is usually considered to be the principal alkaloid in commercial tobaccos, including, *N. tabacum* and *N. rustica*, with few exceptions [Jeffrey & Tso (88); Tso & Jeffrey (198)]. However, nornicotine appeared to be the main alkaloid in more species of *Nicotiana* than did nicotine, and anabasine was the third most important [Smith & Smith (162)]. Many alkaloids have been identified in fermented tobacco (cigar)

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by Frankenburg and his associates (55, 57) and different types of tobacco by Tso & Jeffrey (88, 198). Their significance in synthesis and breakdown of alkaloid in the plant is unknown.

In addition to nicotine, nornicotine, anabasine, and myosmine, green tissue contained amounts of other 3-pyridyl compounds which, collectively, were sometimes equal to, or even greater than, the major alkaloids [Tso & Jeffrey (200)]. The amount of each was too minute for identification.

Many variables affect the quality and quantity of alkaloid present in tobacco plants. Among these are nutrients (60), species or varieties (88, 100), climate (18, 53), soil condition and field practices (60), stage of growth (147, 200), and radiation (152). Many chemicals also affect alkaloid formation (138, 199, 236).

Nicotine is not found in dormant *N. tabacum* seed (125, 140, 200) but is found in seeds of *N. rustica* (125, 157) and *N. glutinosa* (200). Codonnis (36) observed that the tobacco embryo is free of nicotine until four to seven days after germination when the rootlets start to emit protuberances, at which time nicotine appeared in the zone just above them. Il'in (84) observed the synthesis of nicotine-C^{14} by young tobacco seedlings.

Grafts of tobacco with other plants, especially tomato, have been used extensively in studies of alkaloid synthesis and transformation. Neither formation nor translocation of alkaloids [Tso & Jeffrey (202)] was affected seriously, although other slight changes in chemical composition were reported (70). Previous studies [Mothes (123)] led to the view that nicotine is formed in the tobacco root and translocated to the top through xylem, that nornicotine is the demethylation product of nicotine in the top of the plant, and that anabasine can be formed in both root and shoot. Recent work (20, 115, 201) does not entirely agree with these previous findings.

Mothes (123) questioned the work of Mashkovtsev & Sirotenko (115) on embryo grafting on two points: first, that the tomato plant could form nicotine [Wahl (226)], and second, that embryo grafting easily and frequently led to formation of roots in the scion which penetrate the stem of the stock. Not every tomato strain produces nicotine [Tso & Jeffrey (201)], however, and even Wahl's results (226) showed only about 1 to 2 p.p.m. of nicotine produced on a fresh-weight basis, while on another tomato strain, nicotine could not be found though considerable quantities were found in tobacco scions on these roots. Furthermore, Trapp (197) indicated that adventitious roots may be free of nicotine, if not in contact with a supply of nitrogen. In tomato scion on tobacco stock grafts transferred to N^{15}-containing solution, Tso & Jeffrey (201) found the percent of N^{15} excess of nornicotine was higher than that of nicotine in both root and shoot, indicating that nornicotine can be formed independently of nicotine. In reverse grafts transferred to N^{15}-containing nutrient solution, the nicotine and nornicotine in the scions contained over 6 per cent excess N^{15}, indicating alkaloid synthesis without tobacco roots. Excised leaves in

N^{15} nutrient solution increased slightly in nicotine free from excess N^{15}. They believe, therefore, that alkaloid synthesis is a stepwise process and may occur in the top of the plant if the necessary precursors are present. The root is still considered the major site of nicotine synthesis and the source of most, if not all, of the nicotine precursors.

A number of studies have been conducted on the steps involved in nicotine biosynthesis. Although the carboxyl-C^{14} of nicotinic acid or its ethyl ester does not enter into nicotine biogenesis [Dawson, Christman & Anderson (39)]; the pyridine ring of nicotinic acid is reported to be incorporated into tobacco root nicotine [Dawson, Christman & Anderson (40)] as well as nicotine of the intact tobacco plant [Tso & Jeffrey (202]. However, these results did not explain the mechanism of pyridine ring formation, per se. Since nicotinic acid is formed in other plants from tryptophan (5, 16, 122), possibly via 3-hydroxyanthranilic acid, and is also a precursor of trigonelline some investigators believe the same pathway may be important in nicotine synthesis. Bowden (21) stated, however, that nicotine obtained from tobacco plants fed tryptophan was found to exhibit no radioactivity. Trigonelline is formed from nicotinic acid by the pea plant [Zeijlemaker (238)] but that formed from tryptophan-3-C^{14}, was inactive [Leete, Marion & Spenser (104)]. Aronoff (10, 11), on feeding 3-hydroxyanthranilic acid-carboxyl-C^{14} to excised pea tops, could not detect radiotrigonelline, although this system converts nicotinic acid rapidly to trigonelline. Thus, it seems unlikely that the formation of the pyridine ring can occur through the tryptophan, 3-hydroxyanthranilic acid, nicotine acid, or trigonelline system (9).

The conversion of lysine to pipecolic acid occurred in both plants and animals [Grobbelaar & Steward (73)]. Leete (101, 103), however, reported that nicotine obtained from *N. tabacum* which was fed lysine-2-C^{14} was found inactive.

The role of either ornithine or proline in the formation of nicotine has long been postulated [Dewey (44)]. Dewey, Byerrum & Ball (46) found that ornithine-2-C^{14} is incorporated into the pyrrolidine ring of nicotine in *N. rustica*. Leete (102) obtained similar results and suggested that ornithine is converted into a symmetrical compound, such as putrescine, thus giving rise to two differently labeled 4-aminobutanals through oxidative deamination. These 4-aminobutanals could serve as precursors of pyrrolidine ring in nicotine formation. Dewey (44) recently found radioactive nicotine in *N. rustica* var. *humilis* on feeding leucine-2-C^{14} in nutrient solution. Part, if not all, of the C^{14} was located in the pyrrolidine ring of nicotine, and Dewey suggested that ornithine and leucine are degraded to succinic acid before incorporation into nicotine.

Aronoff (11) reported that feeding lysine-2-C^{14} or hydroxylysine-C^{14} to excised *N. glauca* leaves resulted in an insignificant conversion to anabasine. Leete (103), however, obtained radioactive anabasine by feeding

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lysine-2-C^{14} to *N. glauca* and stated that all the C^{14} resided in the α-C atom of the piperidine ring attached to the pyridine ring.

Byerrum and his co-workers in successive publications list various compounds as donors of the methyl group in nicotine (24, 26 to 29). Dewey (44) compared relative rates of methylation to form nicotine by various compounds, formaldehyde being found most effective. The C^{14} and deuterium doubly labeled methyl group of methionine gave rise to the methyl group of nicotine through transmethylation [Dewey, Byerrum & Ball (45)].

Aronoff (10) found that anabasine formed during photosynthesis in $C^{14}O_2$ is uniformly labeled. He also stated that glucose-U-C^{14} may be used as an ultimate precursor for anabasine.

As already mentioned, it is well known that nornicotine can arise from nicotine [Griffith, Valleau & Stockes (72); Wada (225)]. Tso & Jeffrey (202), however, found that nornicotine can be formed independently of nicotine (201) and indicated the possible interconversions among major alkaloids.

Pigments.—The chlorophyll content of green leaves may vary from 0.5 to 4 per cent [Frankenburg (56)]. The total amount of the yellow pigments, carotene and xanthophyll, is about one-fifth to one-third that of chlorophyll in mature tobacco leaves. The quantity of β-carotene found by Jensen (90) in Pennsylvania cigar tobacco leaf was 0.014 per cent on a dry-weight basis. Weybrew (229) found that carotene from tobacco leaves is composed of a mixture of β-carotene and neo-β-carotene in the proportion of 68: 32, while xanthophyll contained 60 parts lutein, 22 parts neo-xanthin, and 18 parts violaxanthin.

Griffith, Valleau & Jeffrey (71) found that leaf thickness contributed half of the differences in pigment concentration of the dark and burly tobaccos. They reported that the average ratio by weight of total chlorophyll to carotene of 18 varieties studied was 19.75, which is very close to the ratio that one would expect if nine molecules of chlorophyll-*a* and three molecules of chlorophyll-*b* were associated in the same protein group with one molecule of carotene. Wolf (233) found that chlorophyll varied from 0.54 to 1.66 mg. per gm. of fresh tobacco leaf. Flue-cured tobacco had a higher content than Turkish, and burley had the lowest. Chlorophyll-*a* decreased more rapidly than the *b* form during ripening and yellowing of the leaves. Jeffrey & Griffith (87) reported, however, that the rate of loss of chlorophyll in the top leaves was not greatly affected by maturity. During normal curing, chlorophyll decreases rapidly.

Other organic substances.—Rutin was found in several varieties of *N. rustica, N. tabacum* and *N. glauca* (15), and in fresh flowers of *N. tabacum* [Nio & Wada (133)]. Rutin and chlorogenic acid were most abundant of the polyphenols (41, 153). Roberts & Wood (153) were unable to detect caffeic acid and catechins, but reported the presence of a trace of isoquercitrin. Howard and co-workers (82) found 0.0025 per cent isoquercitrin in

air-dried burley tobacco. The amount of polyphenols in tobacco leaves is lower the higher the leaves are located on the stem [Middelburg (120)].

The disappearance, during curing, of the flavonoids found in green leaves is probably due to oxidation and polymerization of polyphenols and other phenolic compounds [Frankenburg (55)]. Frankenburg suggests that the yellow pigments, flavonoids, chlorogenic acid, and related polyphenolic compounds may participate in oxidation of amino acids to form brown pigments during curing.

Thiamine, pantothenic acid, riboflavin, and nicotinic acid were found in cigar tobacco by Jensen (90). Free purines, adenine and guanine, were reported present in certain types of tobaccos by Michl & Kuhn (119). Vlitos and co-workers (219) report an unidentified indole hormone in Maryland and Mammoth tobacco, but failed to detect the presence of 3-indoleacetic acid.

Enzymes.—Amylase, invertase, and peroxidase content of leaves increased gradually with maturity, whereas catalase slowly decreased. Topping increased all but catalase [Nakai & Inaba (128)]. An increase in weight and catalase activity was found when the flower buds were developing [Dobrunov (47)]. Scarascia & Venezian (155) observed the increase in seed of carbonic-anhydrase with increasing age and the disappearance of polyphenol-oxidase when the physiological ripening stage of the seed was reached.

Oxidase and catalase activity displayed diurnal variation [Tombesi (192)]. Amylase decreased with illumination [Veuter (206)]. Oxidase was doubled and carbonic-anhydrase decreased by lack of soil moisture, and such leaf remained green on curing [Tombesi (193)]. Low nitrogen resulted in low carbonic-anhydrase and, particularly, catalase; polyphenol-oxidase decreased with low nitrogen and increased with low potassium and phosphorus in trials of Tombesi & Giovannazzi (195).

Phosphorylase occurred in both plastid and cytoplasm suggesting that starch is normally found in the plastids because that is the site of formation of the glucose-1-phosphate substrate [Madison (114)]. Leaf and root possessed very low cellulase activity (196). Excretion of enzymes, particularly amylase and invertase, from the root has been observed [Krasil'nikov (97)].

Etiolated tobacco contained an enzyme activated by light which oxidized glycolic to glyoxylic acid [Tolbert & Burris (190)]. Tobacco sap, salt-precipitated enzyme, and the lyophilized preparation each oxidized glycolic acid to formic acid and CO_2 via the intermediate glyoxylic acid [Tolbert, Clagett & Burris (191)]. The glyoxylic acid-glutamic acid transaminase has been found in the tobacco leaf [Wilson, King & Burris (232)]. The isolation and action of crystalline glyoxylic acid reductase reported by Zelitch (239) suggested that it plays a role in the respiration of green leaves.

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Cytochrome oxidase was reported to be associated with the mitochondrial fraction of leaf homogenates [McClendon (108)]. It was suggested by Vickery (208) that the cytochrome-cytochrome oxidase system is involved in the mechanism of anion uptake.

Nakai & Inaba (128) followed enzymic activities during flue-curing and found amylase, invertase, catalase, and peroxidase activities of leaf tobacco became high at an early stage and decreased later. The activity of amylase increased threefold, invertase remained unchanged and catalase decreased to less than one-tenth, compared to the fresh leaf. Mothes *et al.* (124) noted that some enzymatic differences between root and leaf were not inherent in the cell but resulted from the effects of light and darkness.

Tricarboxylic-acid cycle.—Pucher & Vickery (143) noted that the organic acids of the tobacco plant include many of those participating in the Krebs cycle. Many intensive studies by Vickery and co-workers (208, 213) have revealed the essentially dynamic status of the organic acids. Similar results were obtained by Zbinovsky & Burris (237). A unifying concept proposed by Dawson (38) would make the Krebs cycle the basis for inherited and culturally induced modifications in composition.

Glock & Jensen (67) identified the presence in young tobacco seedlings of *d*-ketoglutaric, glyoxylic, and pyruvic acids. Acetic acid has been reported on by Hoskin and co-workers (81). However, not all of the organic acids of the Krebs cycle and the enzymes functioning have been reported in tobacco to date.

It is not surprising, therefore, that considerable difference in opinion exists on the presence of an operating Krebs cycle in the tobacco plant. Eichenberger (50) had obtained a respiratory quotient greater than one, and interpreted this to mean the existence of a Krebs cycle. Frey-Wyssling & Waltz (59) consider the same evidence implies nonexistence of a direct relation in metabolism between organic acids and carbohydrates.

Curing of Tobacco

The changes undergone by fresh leaves when hung in the barn under regulated conditions of temperature and humidity are referred to as "curing" (60). It is a vital process and falls into the category of starvation phenomena or inanition [Bacon, Wenger & Bullock (14)] of excised plant parts. There is a voluminous literature which can only be indicated very briefly, since much of the information has been reviewed in the section on organic metabolism. Its purpose is to produce dried leaf of suitable physical properties and chemical composition. Respiratory losses, involving hydrolysis and oxidative deaminization, may lead to a 20 per cent decrease in dry matter with the slower stalk-curing method. Various regimes of ventilation, temperature and humidity control are employed to achieve results considered desirable for different types of tobacco. Many leaves of stalk-cured tobacco do not become dry for several weeks under natural

temperatures and close spacing in the barn. The primed, or picked, leaves of flue-cured tobacco become bone-dry in three or four days at the elevated temperatures that are employed. Translocation between stalk and leaf takes place in the former method [Jensen (90)], and respiratory changes appear to be more deep-seated as compared to flue-curing. Few enzymes, probably, survive destruction under the finishing temperature of 180°F. used in flue-curing.

Frankenburg (57) summarized very clearly the most conspicuous chemical conversions during curing. The first phase in conversion is dominated by activities of hydrolytic enzymes and occurs in either flue-curing or stalk curing. In this phase, disaccharides and polysaccharides hydrolyze to simple sugars; proteins hydrolyze to amino acids which undergo oxidative deamination; pectins and pentosans are partially hydrolyzed to pectic acid, uronic acid, and methyl alcohol; chlorophyll is converted to unknown substances; and there is little or no loss of dry weight. The second phase of conversion, according to Frankenburg, is dominated by oxidative reactions and takes place only in stalk-cured tobaccos. These conversions include the oxidation of simple sugars to acids, CO_2 and H_2O; the increased oxidative deamination of amino acids to form ammonia and amides, particularly asparagine; the changes in organic acids, including conversion of malic to citric acid and decarboxylations; the oxidation and polymerization of phenols to brown products; a small decrease in alkaloids; and an appreciable loss of dry weight, particularly those cured on the stalk.

Summary

Coverage in this review is necessarily incomplete both with respect to subject matter and publications. Little mention or none has been made, for example, of the physiological aspects of reproduction or anatomy. The emphasis placed on metabolism is intentional and in accord with the present trend of investigations. Quality is an important factor in production and has its basis in the physical and chemical properties of the leaf. Much could be done to modify these properties through cultural, curing and fermentation practices if sufficient information was available to determine desirable effects and the manner in which they could be manipulated. Instrumentation of the physical elements of quality has lagged, but the increasing knowledge of the chemical components of the tobacco leaf and their normal interactions may well lead to additional improvements in the future once they have been correlated with quality.

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MINERAL NUTRITION OF TREE CROPS[1,2]

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Certain problems and relationships in the mineral nutrition of orchard crops are more sharply focused than in annual crops because of the large size of fruit trees, the more complex relationship to climatic and soil factors, or because of the great economic importance of the fruit they produce. In addition, orchard crops present certain unique problems in mineral nutrition because of the methods of propagation commonly used and the great depth of rooting. For example, there is evidence that both rootstock and scion variety or species influence the mineral nutrition of fruit trees. Also, because productive orchard trees require large, healthy root systems which probe more deeply into the subsoil than do most annual crops, they grow in a substrate which encompasses an unusually broad gradient in nutrient availability as well as temperature and physical soil structure. In addition, the ponderous nature of field soil fertility experiments with fruit trees, among other factors, has led to greater emphasis on tissue analysis as a means of evaluating the nutritional status of this very important group of crop plants.

The mineral nutrition of specific fruit crops is dealt with in a voluminous text (52) published in 1954. The present review attempts to draw some generalization from studies concerned with the nutrition of fruit trees, to emphasize more recent studies, and to call attention to certain somewhat unique problems and relationships revealed by these studies. It is hoped that this will be of general interest to workers in the field of mineral nutrition, and will stimulate further research in certain areas.

Tree Behavior

The tree crops, grown under field conditions, are subject to a number of mineral deficiencies. These deficiencies influence tree growth, tissue composition, fruit production, and fruit quality. Prevalence of deficiencies may vary from tree crop to tree crop and from location to location, but symptoms of deficiencies of various nutrients are common in most fruit-growing regions of the world.

Nitrogen.—It has been established for most tree crops under most conditions that sooner or later it is necessary to add nitrogen fertilizers to maintain satisfactory production. Recent studies are aimed at a more controlled use of nitrogen in relation to fruiting. This involves studies on meth-

[1] The survey of literature pertaining to this review was concluded in August, 1957.

[2] Contribution No. 992, University of California Citrus Experiment Station, Riverside, California.

ods of application, sources of nitrogen, amounts of nitrogen, and time of application. A method of application now receiving considerable attention involves the use of urea as a foliage spray. Urea is less likely to cause leaf damage than other sources of nitrogen applied as a spray (98). The early work on urea foliage sprays has been reviewed by Boynton (26). Urea is absorbed readily by the foliage of citrus (111), apple (54), banana (89), coffee and cacao (40), but not by the foliage of the stone fruits (160). Jones & Steinacker (112) report a "yellow-tip" on Valencia orange leaves as a result of urea sprays. This effect was shown to be biuret injury and soil applications of biuret produced similar toxic symptoms on citrus (114). Also, biruet has been found to be toxic to avocado as well as citrus seedlings when applied in a nutrient solution (96); it is also toxic to pineapple (180), tung (74), and sour cherry (228). Biuret toxicity has not been reported on apples. Why biuret is toxic to some plants and not to others is not known. Biuret is nitrified in the soil (91) but at a slower rate than is urea or ammonium sulfate (210). Most urea contains some biuret, resulting from the partial decomposition of the urea to biuret at the temperatures encountered in the manufacture and processing of urea (210). Urea containing no more than 0.25 per cent biuret is commercially safe, at the rate of 7½ pounds urea per 100 gallons, as a foliage spray on citrus (116). Biuret tolerance levels have not been established for other tree crops.

Some studies indicate that nutrients are absorbed through the bark of fruit trees but, insofar as the macronutrients are concerned, the amounts are small (102, 220).

Fruit trees have been grown commercially with practically every known source of nitrogen. Studies with citrus indicate that plants thrive about equally well on either ammoniacal or nitrate nitrogen (150, 190, 202, 223, 226), except in extremely acid soils (150, 223). Parker & Jones (150) have shown that nitrogen source, through physical and chemical effects on the soil, can become highly limiting to production. Under most field conditions favorable for tree growth, nitrification proceeds rapidly so that comparison of NH_4^+ and NO_3^- nitrogen sources cannot be made. In general, NH_4^+ nutrition differs from NO_3^- nutrition in three main ways: (*a*) the demand for oxygen to the roots is increased with NH_4^+ nutrition, (*b*) competition for absorption of cations other than NH_4^+ may be detrimental to growth if supply of other cations is low, and (*c*) there may be indirect effects associated with shifts in pH of the medium (144). Smith (205) grew sweet orange seedlings under carefully controlled conditions for periods up to 15 months in aerated solutions with N supplied as $(NH_4)_2SO_4$. Such plants were healthy and essentially equal to others grown with all or part of their N supply from NO_3^- provided the development of excess acidity was prevented. Somewhat greater root development occurred when NO_3^- was the source of nitrogen. Wallace & Mueller (234) in a study with rough lemon cuttings using nitrogen-15 isotope techniques found that, generally, ammonium was absorbed more rapidly than nitrate nitrogen. The average

absorption ratio was 1.84. Absorption of NH_4^+-nitrogen tended to increase with increasing pH, but the absorption of NO_3^--nitrogen decreased with increasing pH only at the highest application rate. The most pronounced effect of pH for both sources was at 8 m. eq. but a differential pH effect was absent at 2 m. eq. of N per liter. Wander & Sites (240) found that variations in pH had little or no effect on NO_3^- absorption and that NH_4^+ was more readily absorbed when the pH was maintained at 5.8. Wallace & Ashcroft (233) report that urea reacts much like NH_4^+-nitrogen on rough lemon cuttings even though it is absorbed as urea. Under conditions favorable for nitrification, the source of nitrogen, NH_4^+ or NO_3^-, appears to be of little direct importance to tree crops; however, the secondary side effects of nitrogen source may be of highest concern (119, 150).

The amount of nitrogen required by tree crops and the time of application have had considerable study during the last decade. It appears that nitrogen fertilization can be overdone. Weeks & Southwick (244) found that McIntosh apple trees which were grown at a relatively high level of nitrogen, as indicated by leaf nitrogen, did not maintain annual production as well as did trees of low nitrogen level. Boynton & Anderson (27) report an increase in yields of McIntosh apples with increasing nitrogen, two years out of four, but there was a greater loss from preharvest drop and a delay in red color development of the fruit from the high nitrogen trees. In another report on McIntosh apples, Boynton (22) states that total yield was significantly increased by the nitrogen increment from 0.5 to 1.0 lb. but not by the increment from 1 to 1.5 lb. The average percentage of the fruit sample which was graded fancy was significantly decreased, however, by both increments of nitrogen. With young Elberta peach trees Ritter (174) found that only the first increment of nitrogen was effective in increasing growth and production. Oranges, like other tree crops, require nitrogen fertilization for production. Present grower practices in some areas appear to involve excessive application of nitrogen. Jones & Embleton (115), in a number of experiments with Valencia oranges, found no effects on yields after omitting nitrogen for a period of two to six years. Reuther & Smith (169), working on the sandy soils of Florida with Hamlin and Parson Brown oranges, found that in general, neither the time of application nor the number of applications of a given adequate annual rate of nitrogen fertilization profoundly affects yield or tree vigor.

Phosphorus—Response of fruit trees in commercial orchards to phosphorus applications are rare (52). Some response has been obtained with tung as reported by Drosdoff *et al.* (72). In a phosphorus-deficient Aiken soil in California, Lilleland *et al.* (129) found that eighteen different annual crops failed to make satisfactory growth unless phosphate was added. On this same soil, established fruit trees—cherry, prune, almond, peach, walnut, and filbert—showed no response to phosphate. In recent years, responses to phosphate applications have been reported for citrus [Williams & Gates (249); Bouma (18); Aldrich & Haas (1); Aldrich & Coony (2); Coony

& Aldrich (56); and Embleton *et al.* (80)]. Embleton describes the development of phosphorus deficiency in Valencia orange trees. The first visible symptoms are a characteristic type of deterioration in fruit quality. The details are described later in the section on fruit quality. After the fruit quality begins to deteriorate, the foliage density becomes progressively thinner because fewer and smaller leaves are produced which drop prematurely. Accompanying this reduction in foliage density is a decrease in yield. A weak spring flush of growth is the only one to appear, and in the late fall and winter, this foliage takes on a bronzed cast. Some twig dieback is present. Embleton *et al.* (78) report that phosphate applications to these trees increase yields and reduce fruit sizes.

Even though some responses to phosphate reported have been dramatic, such response in tree crops is still rare. Chapman & Vanselow (51) report that probably less than 10 per cent of California citrus groves are in need of, or likely to be in need of, supplemental phosphate for some time. They point out that citrus trees can receive adequate phosphorus from many soils in which field and garden crops fail for lack of this element and also that there is an accumulation of phosphorus in many citrus orchard soils from the use of mixed fertilizers and manures. In a long-term experiment with oranges in California, Parker & Jones (150) found no influence of phosphorus application on yields. This result indicates that the soil was well supplied with phosphorus. In these same experiments, it was found that after 28 years on plots with no phosphate application, the loss of P from the highest-yielding plot by removal with the fruit amounted to only about 2 per cent of the total P in the 0 to 36 in. layer of soil and that about 7 per cent of the total P had undergone absorption and redistribution by the trees (156, 157). In the process of redistribution, the P has been deposited under the tree as a result of leaf fall. As a result of this deposition, each tree has an area of soil surrounding the trunk in which the total and available P is higher than the rest of the soil area. The phosphorus that accumulates under the tree, presumably, is in a form more available to plants than the native form. Thus it can be reasoned that phosphorus available to the trees may even increase with time.

Phosphorus applied to the soil moves downward very slowly. In California experiments, it was found that more than 60 per cent of the accumulation of P from the application, over a 28-year period, of phosphatic or organic materials, was in the top 6 in. of the soil and more than 80 per cent was in the top 12 in. Only a very small amount of applied phosphorus penetrated beyond 24 in. (156). Similar evidence of the strong tendency of applied phosphorus to accumulate in the top soil in orchards has been reported by others (164, 204, 208). Thus, heavy phosphate fertilization in orchards over a period of years is likely to cause a sharp gradient with depth in the supply of phosphorus available to the roots of fruit trees.

Potassium.—The level of potassium nutrition based on leaf analysis may vary over a relatively wide range with no observable effects on tree

condition or on production. Lilleland & Brown (128), in a survey of 130 peach orchards, found a range of leaf potassium from 0.6 to 3.4 per cent with no observable differences in tree condition that could be related to potassium level. Likewise, Jones & Cree (113) found no consistent relationship between tree condition and potassium level, in a survey of some 440 California Valencia and Washington navel orange orchards in which the leaf potassium varied from 0.37 to 1.66 per cent. Studies with peaches and prunes indicate that crop load is inversely related to the potassium content of leaves (127, 154). More recent studies with potassium have been conducted in relation to fruit size, especially with citrus. Smith & Reuther (198) reported an increase in fruit size with an increase in leaf potassium. Parker & Jones (149), in a long-term experiment with Washington navel oranges, found that the correlation between fruit size and leaf potassium was positive and curvilinear up to about 1.3 per cent leaf potassium. The response in fruit size occurred at levels of potassium nutrition, as indicated by leaf analyses, which are higher than those which influence fruit setting, preharvest drop, or other recognized visible symptoms of potassium deficiency. However, Jones & Cree (113) in a survey of some 440 orange groves, found that the positive correlation between leaf potassium and fruit size did not always hold; in fact, in some locations it was negative. This lack of consistency was found by Jones & Embleton (117) to be related to other limiting factors, such as water or magnesium supply.

Calcium.—From a nutritional standpoint, no field response to added calcium has been reported on tree crops. Most soils are well supplied with calcium and calcium salts are frequently applied to maintain a favorable pH or soil structure or both. In pot cultures with young lemon trees, Jones *et al.* (121) found toxicity symtoms of Na and K, rather than deficiency symptoms of Ca, when the exchangeable Ca was relatively low and exchangeable Na and K relatively high. In sand and water cultures, Brusca & Haas (30) found a reduction in leaf size in citrus when the nutrient solution was low in calcium.

Magnesium.—Magnesium deficiency is common in tree crops in many parts of the world (52). In many cases this deficiency can be related to cultural practices. Data obtained by Pratt & Harding (158) by the analysis of soil samples taken in 1927 and in 1955 from plots of the long-term fertility trial with oranges at Riverside, California, show that there were relatively large losses of magnesium from the soil during this time interval where only irrigation water was applied. The losses of magnesium increased with an increase in the amount of fertilizers and gypsum added to the soil. The irrigation water had a ratio of calcium to magnesium of 3:1. This ratio of calcium to magnesium was conducive to magnesium loss from the soil. Bingham *et al.* (13) found that a K/Mg ratio in the 18- to 30-in. depth of more than 0.4 to 0.5 was related to magnesium deficiency in orange trees. The depth, of course, would vary with the depth of rooting. It appears that future work, especially in irrigated areas or where potash fertilizers are

needed, should be directed toward methods of maintaining magnesium in the soil.

Micronutrients.—In one location or another, for various tree crops, deficiencies have been reported of boron, copper, iron, manganese, molybdenum, and zinc. Most of these are relatively simple to correct by foliage sprays. The most recently reported deficiency is that of molybdenum on citrus in Florida. Stewart & Leonard (213) reported that molybdenum deficiency, which occurs as a yellow spotting on citrus leaves, could be corrected with sprays of Na_2MoO_4 or $(NH_4)_2MoO_4$ at the rate of 0.1 to 0.4 ounces per tree applied in 10 gallons of water. They also reported that grapefruit on soil at pH 4.2 were Mo deficient and had 0.03 p.p.m. Mo in the leaves (211). When this soil was limed to pH 5.6 the deficiency was corrected and the molybdenum in the leaves raised to 0.06 p.p.m.

Iron deficiency is the most difficult to correct. It is found in almost every fruit-growing area in the world. It occurs on both alkaline and acid soils. When it occurs on alkaline soils it is referred to as lime-induced chlorosis. On the acid soils of Florida, Stewart & Leonard (126, 212) corrected iron deficiency in citrus by soil applications of iron chelates. They found that 20 gm. of iron per tree, as the iron salt of ethylenediamine tetraacetic acid (EDTA), was effective. In the calcareous citrus soils (pH 7 to 8) of California, Wallihan *et al.* (238) found no benefit from soil applications of EDTA or from various other chelating agents. Experiments in some European fruit-growing areas indicate that certain chelates may have some promise for controlling chlorosis on calcareous soils (17, 92). Many different chelates are now available and are being tried on various crops. They have been discussed in a recent symposium, edited by Wallace (232).

Mineral Excesses

Excesses of nutrient and non-nutrient mineral elements can and do occur under both irrigated and nonirrigated production. The former are associated with irrigation water and the latter with other cultural practices. From an agricultural standpoint, rainfall is free of salts but all irrigation waters contain salts dissolved from the rocks and soils through which the water moves. The excellent report on arid lands edited by White (246) discusses many of the problems of excess salts in irrigation water. It is clear that to establish a permanent irrigation agriculture—be it with tree crops or otherwise—methods for handling mineral excesses must be developed.

In irrigated soils, two types of excesses are likely to occur: (*a*) excess total salt (saline soil condition), and (*b*) excess of a specific element such as boron. Both types may occur together or they may occur separately. Where rainfall is adequate, saline conditions are not likely to develop but an excess of a specific element may occur.

In a long-term experiment with oranges at Riverside, California, Harding *et al.* (101) found a highly significant negative correlation between yield of fruit and soil salinity. In this same experiment, Jones *et al.*

(119) reported a highly significant positive correlation between yield of fruit and water intake by the soil. In this experiment the effects of salinity and the effects of the lack of water cannot be separately determined but it is clear that where water infiltration is poor, salinity is high and production is low. Under sprinkler irrigation with water of 700 p.p.m. or more of soluble salts, Harding *et al.* (100) found damage to citrus through leaf absorption of the salts. Pearson *et al.* (152), in a study of the influence of salinity and water table on the growth of young grapefruit trees, found that the salinity factor accounted for approximately 90 per cent of the variance in growth. A conductivity value of approximately four millimhos in the saturation extract was associated with a 50 per cent decrease in cross-sectional area of the trunk. Only in the absence of salinity was there a highly significant reduction in the fresh weight of the tops attributed to a fluctuating water table. In a survey of 22 mature navel and Valencia orange groves in California, Cahoon *et al.* (33) found an inverse relation between yields and root density in the top 3 ft. of soil. The lowest root density occurred in the irrigated middles. This is the location of build-up of salinity (101). However, factors other than salinity are also involved (5).

In general, tree fruits have a low tolerance to the high osmotic pressures of saline substrates but specific toxic effects are also common. Hayward *et al.* (104) related the severity of burning and injury of peach leaves to the accumulation of chloride ions in them. Lilleland *et al.* (130) observed that the severity of tip-burn of almond leaves was directly related to their sodium content. In a sand culture study with peach, apricot, plum, prune, nonpareil almond, and Texas almond on saline substrates, Brown *et al.* (29) found that the effects of treatment reflected a greater influence of specific ions than of the osmotic pressure of the solutions. Bernstein *et al.* (12), in a field plot study with the same six species, found that trees made only one-third as much growth on moderately saline plots as on nonsaline plots. They determined that about half of the reduction in growth was due to chloride toxicity, and the other half to increased osmotic pressure of the saline soil solution. Observations by the authors indicate that the avocado is very sensitive to chloride and that leaf burn will occur on nonsaline soils when the chloride in the leaf reaches 0.25 per cent of the dry weight. Cooper & Gorton (60) found that, with grapefruit, manifestations of toxicity were closely related to an excess in chloride accumulation over the total cation increases. In a greenhouse study with young budded lemon trees, Jones *et al.* (121) found that growth was decreased as the percentage of sodium or potassium or both on the exchange complex was increased.

Those elements that are fixed on the exchange complex, or otherwise, in the soil are not readily leached and may accumulate to harmful levels with continued use. Unpublished data of the authors from the long-term fertility trial in Riverside, indicate that potassium now may have reached a toxic level in some plots. In the production of citrus in Florida, it has

been the practice for many years to add heavy metals (zinc, manganese, copper) as foliage sprays and in mixed fertilizers. These practices have resulted in rather large accumulations of these metals in the soil and in considerable copper toxicity. In a number of papers, Reuther & Smith (167, 168), Smith (195, 203), and Smith & Specht (197) have reported the magnitude of the problem and corrective measures. Here the toxicity problem has developed through the overapplication of a required nutrient element that is not readily leached from the soil even under high rainfall conditions (172, 239). Likewise in many orchards in many locations, arsenic has also built up to toxic levels in soils from spray residues. Lindner *et al.* (131, 132) report arsenic damage on peaches and point out that the stone fruits are sensitive to arsenic, while pears and apples are more tolerant. The continued addition to orchard soils of such materials that may accumulate to toxic levels should be given serious consideration in orchard practices.

Boron may be in excess, especially in irrigated areas. Chapman & Vanselow (50) point out that B excess commonly results when irrigation water contains more than 0.5 p.p.m. boron. Where the concentration is greater than 1.0 p.p.m., boron toxicity is apt to be fairly pronounced and waters containing more than 2 p.p.m. boron are generally regarded as unfit for citrus irrigation. Citrus leaves containing 200 p.p.m. boron on a dry weight basis may show moderate leaf symptoms. Aldrich *et al.* (3) report an instance of lithium toxicity in citrus. The trees were irrigated with water containing from 0.045 to 0.08 p.p.m. lithium. Leaves, on a dry weight basis, contained 12, 14, 23, and 14 p.p.m. lithium for lemon, grapefruit, navel orange, and Valencia orange, respectively. Stewart & Leonard report that perchlorates are toxic to citrus (214). Perchlorates occur as traces in some natural nitrate materials.

Automobile exhausts and industrial wastes have added pollutants to the atmosphere which are, in many instances, toxic to plants. Fluorine injury has been reported by DeOng (69) on apricots, by Johnson *et al.* (108) on prunes, and by Shaw *et al.* (182) on ponderosa pine. Kaudy *et al.* (122) found from one p.p.m. fluorine or less up to 211 p.p.m. in citrus leaves, depending on the distance from the source of contamination. Taylor *et al.* (218) found that "smog" greatly reduced the growth of avocado seedlings. These pollutants will be of more and more concern to the producer of tree fruits as time goes on unless methods of abatement are found.

Fruit Quality

A great deal of progress has been made during the past 25 or 30 years in isolating some of the important components of fruit quality, and defining these in quantitative terms using physical and chemical measurements as indices. The indices of fruit quality which have been studied most extensively are size (weight, diameter, number per box), rind color, texture and thickness of rind or skin, flesh color, texture and firmness, juice

color, juice content, and total soluble solids, sugar, acid, vitamin, and oil content of flesh or juice. Very little progress has been made in defining in quantitative terms that aspect of fruit quality related to the "bouquet," or the distinctive flavor, imparted by certain essential oils and other flavor constituents present in minute amounts.

Many fruit industries have adopted minimum quality standards for various grades offered to consumers, some on a purely voluntary basis, but most standards are upheld by legislation with supporting inspection. The standards defining the size and other indices of quality vary greatly, both quantitatively and qualitatively, among classes and even among varieties within classes, of tree fruits. For example, quality standards for dessert apples emphasize size, color, and firmness of flesh, while those for oranges include size, color, juice content, and total soluble solids and acid, and the ratio of these, in the juice.

The fact that fruit quality (as defined by standards set largely by consumer preferences) affects profits to growers, has stimulated a great deal of research, both fundamental and applied, on the effect of various production practices and other factors on fruit quality, as well as total yield of orchard trees. A large body of literature indicates that the nutrient status of orchard trees, among many other factors, has important effects on the gross morphology, and some physical and chemical aspects of the tissues of the fruit they produce.

Nitrogen.—The rate of growth and ultimate size of most fruits at harvest time are conditioned by a number of factors, among the most important of which are prevailing temperature, moisture supply in the soil, tree health and vigor, crop density, and nutritional status of the tree. The bulk of the evidence available indicates that, within the range sustaining economic production, the nitrogen level in the tree does not ordinarily exert any direct, primary influence on fruit size, but may influence it indirectly through its effect on crop density. Heavy nitrogen fertilization, resulting in an increase in the number of fruits set, may cause a decrease in the average size of fruit at harvest time (11, 24, 198).

The color of the rind of most fruits used for dessert purposes is a very important component of market quality. Hence the influence of various factors on skin pigmentation of fruits has received widespread attention by horticulturists and physiologists. In general, a level of nitrogen nutrition high enough to insure maximum fruit yields from orchards usually produces a high proportion of poorly-colored fruit. Conversely, a level of nitrogen nutrition sufficiently low to limit yield slightly is usually associated with a high proportion of well-colored fruit of good market quality.

The apple provides an excellent example of this color response to level of nitrogen nutrition. A large body of experimental evidence has accumulated, indicating that development of red and yellow color of the skin shows a strong inverse relation to the nitrogen level in the tree at harvest time

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(10, 19, 22, 23, 24, 82, 185, 243). The nitrogen level in the apple tree during the two or three months before harvest appears to be the most influential on fruit color development. As a result, the timing of nitrogen applications, particularly with urea foliage sprays in the case of McIntosh apples in the Northeast, has been found to influence fruit color (22, 25, 82). Fruit color of the pear responds to the level of nitrogen nutrition in much the same way as apples (25, 145). Likewise, numerous studies show that the development of red and yellow coloration of peaches is inversely related to the level of nitrogen in the trees producing them (11, 42, 85, 103, 161, 181). A similar relation of nitrogen nutrition to red color of cherries has been noted (209, 236). The nitrogen status of some stone fruits (plum, prune, apricot) ordinarily does not affect the color of the fruit (132). However, an experiment on Italian prunes in New York showed that both skin and flesh color were influenced by nitrogen fertilization (35).

With oranges and grapefruit, a high nitrogen level in the tree in the three- or four-month period before the fruit is mature is associated with a delay in the development of the desirable orange or yellow color of the rind (46, 105, 109, 123, 139, 140, 165, 198). Spring applications of nitrogen resulted in a delay in "color break" of Parson Brown oranges when compared to fall or summer applications; and nitrogen applied in the fall resulted in an earlier "color break" in the rind of Hamlin oranges than when nitrogen was applied in three applications a year (169). Preliminary findings by the authors (unpublished) indicate that with Valencia oranges in California, summer applications of nitrogen result in a greener fruit color at harvest time than when spring or split applications of nitrogen are made, and that soil applications of nitrogen produce greener fruit than spray applications.

Firmness of flesh is an important index of quality and maturity of some fruits. Boynton (24) cites some examples showing that an increase in the rate of nitrogen applied to apple trees is sometimes associated with softer fruit and that the softer fruit generally have a shorter storage life. More recent reports confirm that fruit from high-nitrogen apple trees are less firm than fruit from low-nitrogen trees (23, 206, 243). Spray applications of nitrogen to apple trees resulted in lower fruit pressure readings than following soil applications (16). It is not surprising, in view of the close relationship to the apple, that pears from high-nitrogen trees are initially softer (145) and soften somewhat more rapidly during the first three months of storage (68) than pears from low-nitrogen trees. Nitrogen level in the peach tree usually does not appear to influence firmness of the flesh of peach fruits (58, 65, 67, 161), as it does in the pome fruits.

No definite trends were found on the effect of differential nitrogen rate applications on soluble solids, acids, or the ratio of soluble solids to acid in apple fruits (10). Urea-sprayed apple trees appear to have a lower percentage of total solids in the fruit than fruit from trees that received soil

applications of nitrogen (16). Soluble solids in peaches appear to decrease with increasing nitrogen (161, 134). Nitrogen sources or timing methods seem to have no significant influence on the soluble solids content of cherries (228). In one experiment with peaches, titratable acidity decreased and the ratio of solids to acid increased with an increase in nitrogen (161). With most citrus fruits, the total soluble solids and citric acid concentrations, the ratio of solids to acid in the juice, and the percentage of juice in the fruit, are indices used in defining quality and maturity standards. Manufacturers of concentrated citrus juices base value of the fruit they purchase largely on these factors, since they determine the yield of final product that can be obtained from a ton of fruit. This has stimulated more research on the effect of various orchard practices on juice quality of citrus than has been the case with any other tree fruit. The effects of nutritional level and fertilizer practices on citrus juice yield and quality have received especially intensive investigation.

A survey of the voluminous literature on citrus cited by Smith & Reuther (198) and others (109, 229, 231) indicates that the total content of soluble solids in the juice is not directly related to the nitrogen level in the trees, since under varying conditions an increase in nitrogen level has been reported variously to increase, decrease, or have no effect on total soluble solids in juice. However, in a majority of the reports, the data show a tendency for an inverse relation between nitrogen level and concentration of solids in the juice. The timing of nitrogen application also appears, in general, to have little consistent effect on the solids content of juice (162, 169, 173, 189).

The majority of pertinent experiments indicates that an increase in nitrogen level in the citrus tree causes an increase in the total acid content of the juice of the fruit (109, 110, 123, 138, 173, 198, 229, 231), but there are minor exceptions (105, 110). Timing of nitrogen applications to citrus trees is, in general, without influence on acidity, except in cases where timing markedly influences the nitrogen level in the tree during the two- or three-month period before the fruit reached maturity (169, 173).

The ratio of total soluble solids to acid in the juice is a factor strongly related to consumer acceptance of citrus fruits and is one important index used to define when citrus is ripe enough to pick for sale. As would be expected from the trends in solids and acids in juice produced by nitrogen fertilization most of the available data indicates that an increase in nitrogen level in the tree is associated with a slight delay in the date that picking maturity is reached, as judged by the ratio of total soluble solids to acid in the juice (109, 123, 165, 166, 173), but there are some exceptions (46, 105, 110). In general, nitrogen timing treatments do not have consistent, strong effects on the ratio of solids to acids in juice (140, 162, 169, 173, 189, 198).

The thickness of the peel of citrus is obviously inversely related to the percentage of juice in the fruit. Because of the fact that a thick peel

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is usually directly related to the coarseness or roughness of the surface texture of the peel, much information has been obtained on factors affecting citrus peel thickness. A smooth skin texture is considered highly desirable. Apparently, nitrogen level in the tree has no direct, primary effect on percentage of juice (reflecting peel thickness) in citrus fruits. Reports from Florida indicate an increase (136, 166, 196), from California and South Africa, a decrease (110, 120, 138, 229, 231), and in one experiment (110), no effect on juiciness from added nitrogen.

Because of the importance of ascorbic acid in human nutrition, and the ease of assaying it, the influence of the nutritional status of fruit trees on ascorbic acid content of fruits has been studied extensively especially with citrus fruits. The evidence available indicates that heavy nitrogen fertilization of apple, peach, and citrus usually produces fruits with lowered ascorbic acid content (28, 109, 120, 142, 198, 235, 250), but exceptions occur (161, 169, 173).

In the avocado and in nut crops the percentage of oil in certain parts of the fruit is a very important aspect of quality. In an experiment with the Fuerte avocado, the highest percentage of oil in the pulp was associated with an intermediate nitrogen level (77), as indicated by leaf analysis. An experiment with pecans indicated that the percentage of oil in the kernel was inversely related to the vegetativeness of the tree. Rate of nitrogen application was one of the factors influencing vegetativeness (81). Very high levels of nitrogen tend to delay maturity and to reduce the filling and oil content of nuts (64). Intensive studies on tung showed that while high-nitrogen treatment increased yield of kernels, the percentage of oil in the kernels in fruits from trees liberally fertilized annually with nitrogen alone declined from year to year and, ultimately, the result was to reduce, instead of increase, the percentage of oil based on the whole fruit. Combining high nitrogen with high potassium resulted in the highest recoverable oil production per acre (72).

Phosphorus.—No definite conclusions can be drawn from the limited evidence available concerning the effect of the level of phosphorus nutrition on color and other quality factors of deciduous fruits (23, 57, 59, 84, 107, 133, 137, 143, 146, 179). However, in North Carolina, peach trees definitely deficient in phosphorus, produce fruit that color and ripen abnormally early, have an unpleasant taste and are misshapen (225).

Orange trees which are deficient in phosphorus may produce some of the following fruit characters: large, coarse-textured fruit with hollow cores, high acid and low solids content of juice, and deep orange color of rind (18, 45, 49, 76, 79, 88).

Phosphate fertilization of citrus trees may produce some effects on fruit quality even when yield is not affected. Many reports show that heavy phosphate fertilization of apparently normal, vigorous citrus trees

results in a slight lowering of total soluble solids, acid and ascorbic acid in the juice, the formation of a thinner peel associated with an increase in juiciness, an increase in the ratio of solids to acids in the juice (hastening of maturity), and a tendency for some varieties to be greener in color (78, 138, 198). Solution culture studies on the Valencia orange indicated that "creasing" or "crease and puff," an undesirable disorder of the peel resulting in poor shipping quality, was increased by a high phosphate level in the substrate (95). Another solution culture study on Washington navel oranges showed no appreciable influence of phosphate level on "creasing" (49). Unpublished field data of the authors show an increase in "creasing" associated with phosphate applications.

Potassium.—Most of the available evidence indicates that apple trees affected with "leaf-scorch" symptoms of potassium deficiency produce fruit which do not color normally as they ripen. Potassium fertilization of such trees usually improves vigor and foliage condition, and markedly improves fruit color. However, potassium added to apple trees having a potassium level in them ample for vigorous growth, does not influence fruit color (24). In a recent field fertilizer experiment with apple trees, the interaction of potassium and nitrogen with fruit color was studied. The data showed that the best colored fruits were produced by low-nitrogen, high-potassium treatments, and the poorest by the high-nitrogen, low-potassium treatments. Multiple regression analysis of the data suggested that at high nitrogen levels in the tree (as indicated by leaf analysis) potassium may be a factor limiting red color development (243).

A survey of the limited data available does not reveal any clear-cut generalization that can be drawn concerning the effect of potassium level in deciduous fruit trees on such factors as firmness of flesh, total soluble solids and acid in the flesh, or time of ripening (52). Potassium-deficient peach trees produce fruits with a marked lack of color in both skin and flesh (107). Potassium fertilization improved color and size of pear fruit (83).

With citrus, the data available indicate that the effects of the level of potassium nutrition on certain aspects of fruit quality are fairly clear-cut. As discussed in the section on Tree Behavior, the level of potassium nutrition of the tree, as indicated by the potassium content of the foliage, usually is directly related to mean fruit size (198). There appears to be no consistent relation between potassium level in trees and content of total soluble solids or ascorbic acid concentration in the juice of fruits produced, but there is consistent evidence from widespread sources, under widely varying conditions, that the level of potassium nutrition is directly related to the acid content of the juice and inversely related to the ratio of solids to acid in the juice (79, 138, 198).

Some reports indicate that peel thickness of citrus is increased, and

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juiciness and orange color of the skin is reduced, by increasing the potassium level in citrus trees (79, 198). "Creasing" of the rind appears to be reduced by heavy potassium fertilization (188, 224).

Potassium appears to have some influence on oil formation in nuts. Several investigators have reported that the application of potassium fertilizers to pecans (191) and tung (72, 125) increased the oil content. In one experiment, potassium and magnesium fertilizers applied together produced a significant increase in oil content of tung fruit, but neither material, when applied separately, produced an improvement in oil content (73).

Calcium and magnesium.—There is not sufficient data available concerning the influence of calcium and magnesium nutrition on quality of deciduous fruits or nuts to draw any meaningful conclusions. However, limited data from experiments with the apple suggest that fruits produced by trees suffering from acute magnesium deficiency fail to ripen normally, are small in size, poorly colored, and lacking in flavor (236). Also, there is some evidence that magnesium fertilization may improve the flesh firmness at picking time (65).

With citrus, however, it has been noted that severe magnesium deficiency is usually associated with abnormally low concentrations of total soluble solids, citric acid, and ascorbic acid in the juice (41, 62, 63, 90, 178, 187, 196). In general, increasing the level of magnesium nutrition in normal, vigorous citrus trees, substantially free of foliage symptoms of magnesium deficiency, has no striking effect on fruit quality (166).

The direct effect of level of calcium nutrition on fruit quality has not been effectively disentangled from all the side effects on availability and uptake of other nutrients that result from the application of most calcium materials to the soil, or that occur upon replacing calcium with other basic cations in nutrient culture solution studies (11, 24, 72, 198). Indeed, most of the effects attributed to increasing the level of potassium nutrition in orchard or controlled nutrient culture studies could be attributed, with almost equal validity, to decreasing the level of calcium nutrition (198). These two elements commonly show an inverse relationship in plant tissues.

Micronutrients.—Almost no information is available concerning the effects of the level of micronutrient nutrition on fruit quality. However, some distinctive symptoms involving fruit quality have been found to be associated with acute deficiencies of certain micronutrients. Peach trees having symptoms of acute zinc deficiency often produce misshapen, pointed fruits with flat cheeks (11). Nuts from zinc-deficient tung trees may be low in oil (72), and from pecan and walnut trees, abnormally small in size (159). Zinc deficiency of citrus is a common disorder of this crop, and has been extensively studied. Fruit from zinc-deficient citrus trees may be small in size, low in juice content, low in acid and ascorbic acid content of the juice and have a pale rind color when mature (41, 147, 148, 178).

Fruits from chlorotic citrus trees in Florida, which suffer from acute iron deficiency (induced by copper toxicity), have a pale rind and juice color, are low in juice content and total soluble solids content in the juice, and tend to mature late (47, 215).

An acute deficiency of manganese in citrus trees is associated with soft fruit and a pale rind color (4, 177, 192). The shells of nuts produced by copper-deficient walnut trees have a light, chalky appearance, and the kernels are badly shrivelled (159).

Copper deficiency of citrus has been studied extensively. Fruit from copper-deficient orange trees have characteristic, brownish, gum-soaked lesions on the rind, and may have gum pockets surrounding and adjacent to the central pith. Young fruit may be light-green in color, and tend to split and drop off. The peel tends to be thick, and the juice somewhat low in total soluble solids, acid and ascorbic acid (47, 90).

In citrus, boron deficiency usually results in gum-soaked spots in the albedo of the fruit, lumpiness and a hard, dry fruit, prone to drop off prematurely (194). Boron deficiency may cause distorted fruit in olives (44), and shrivelled and blotchy fruit in cherries (241); it causes internal or external lesions in apples and the fruit may be distorted and lack acidity and juciness (31, 66). With apricots, boron deficiency is associated with internal browning and corky tissue, external cracking and browning, shrivelling and acute malformation of the fruit surface (132). With European plums, boron-deficient fruits have brown sunken areas in the flesh and color up and drop prematurely (99); boron-deficient pears have shallow depressions in the fruit with corky areas under the depressions (25).

Boron applications to peaches were found to hasten maturity and reduce red coloration of the fruit (248). An excess of boron has been found to hasten maturity and the change from green to yellow ground color of apple fruit (24). Excessive boron applied to peach trees was found to hasten maturity of fruits one to three weeks (53). Slightly toxic levels of boron in citrus trees caused the fruit to have slightly less acid and ascorbic acid in the juice (201).

Leaf Analysis

Orchard fruit and nut trees are usually planted from 30 to 120 trees per acre, and have a commercially-productive life of from 15 to 60 years or more, depending on species, disease, climatic, and soil factors. These basic factors of large size and long life have made field studies on the effects of fertilizer treatments with tree crops inherently ponderous and expensive to maintain. In addition, the large unit size of the plant makes precision in determining yield and growth effects relatively low in experiments with tree crops of manageable size (118). Cumulative effects of previous seasonal and cropping history make it necessary to obtain yield

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records over a number of years before trends can be established with some degree of certainty (151, 155). At a given level of research resources in terms of men and facilities, many more field fertilizer experiments can be handled with annual crops than with orchard crops. This fact usually results in an inadequate sampling of variations in soil conditions in orchard fertilizer experiments in a given area. The large size of fruiting orchard trees, and the five- to six-year period of growth after planting required before fruiting begins, has sharply limited studies involving the measurement of yield and fruit quality with trees growing in pots, or other vessels of artificial culture media, or soil in which a high degree of control can be exercised over mineral nutrient factors.

All of the above factors have made for relatively slow progress in determining the effects of levels of specific mineral nutrients on growth and fruiting of orchard trees. In the last two decades, however, the rate of progress has been appreciably accelerated by the use of tissue analyses as a means of evaluating the nutritional status of orchard trees produced by various fertilizer treatments (48, 72, 170, 171, 237). Development of this tool has been a great step forward, providing a means of assaying results of orchard fertilizer experiments on one type of soil in terms applicable to other soil types. Likewise, it has provided a means of translating responses of fruit trees to mineral nutrient treatment obtained in sand or solution cultures to orchard-grown trees. As a result of these considerations, investigators of the soil fertility problems of orchard crops in particular have emphasized leaf analysis as a basic research tool.

In addition, the development of leaf analysis as an applied tool is progressing rapidly. With such major fruit and nut crops as the apple, peach, tung and orange (11, 24, 72, 106, 170), sufficient leaf analysis data have been obtained from sand and water culture studies, from field fertilizer experiments, and from orchard surveys to formulate tentative standards for classifying leaf composition; these standards are proving very useful in determining the nutritional status of orchard crops. The use of such standards (in many cases in conjunction with soil analysis) is expanding rapidly among extension workers, technical advisors, and consultants in fruit-growing industries. They are proving to be a very useful guide in assessing and correcting faulty mineral nutrition as a factor limiting production and quality of fruit crops. Also, routine leaf analyses are proving very helpful in reducing costs and improving efficiency of fertilizer practices.

Examples of leaf analysis standards which have been proposed are presented in Tables I and II. These are more or less empirical values and are based on a specific type and age of leaf sampled in a standard way. Note that despite the wide difference in plant species and growth habit of tung and citrus, there is a surprising degree of agreement in "marginal range" proposed for tung and the equivalent "low range" proposed for citrus. With most orchard trees, fruit quality, as well as yield, is taken into

TABLE I

TENTATIVE STANDARDS FOR CLASSIFICATION OF THE NUTRIENT STATUS OF VALENCIA ORANGE TREES BASED ON LEAF ANALYSIS

Element	Unit (dry matter basis)	Ranges*				
		Deficient	Low	Optimum	High	Excess
N	%	<1.9	2.0 to 2.3	2.4 to 2.7	2.8 to 3.2	>3.3
P	%	<0.08	0.09 to 0.11	0.12 to 0.16	0.17 to 0.29	>0.3?
K	%	<0.6	0.7 to 1.1	1.2 to 1.7	1.8 to 2.3	>2.4
Ca	%	<1.5?	1.6 to 2.9	3.0 to 5.5	5.6 to 6.9	>7.0?
Mg	%	<0.15	0.16 to 0.29	0.3 to 0.6	0.7 to 1.1	>1.2?
S	%	<0.13	0.14 to 0.19	0.2 to 0.3	0.4 to 0.5?	>0.6?
B	p.p.m.	<20	21 to 40	50 to 150	160 to 260	> 270
Fe	p.p.m.	<35	36 to 59	60 to 120	130 to 200?	> 250?
Mn	p.p.m.	<15	16 to 24	25 to 200?	300 to 500?	>1000?
Zn	p.p.m.	<15	16 to 24	25 to 100?	110 to 200?	> 300?
Cu	p.p.m.	<3.5	3.6 to 4.9	5 to 16?	17 to 22?	> 23
Mo	p.p.m.	<0.05	0.06 to 0.09	0.10 to 0.29?	0.3 to 0.4?	—?
Cl	%	?	?	<0.3	0.4 to 0.6	>0.7?
Na	%	†	—	<0.16	0.17 to 0.24	>0.25?
Li	p.p.m.	†	—	<1?	2 to 9	>10?

* Based on concentration of elements listed in 4- to 7-month-old, bloom-cycle leaves from nonfruiting terminals. Adapted and revised from Reuther & Smith (170).

† These elements are not known to be essential for normal growth of citrus.

consideration in setting leaf standards. Some of the important effects that mineral nutrition may have on fruit quality are reviewed in another section.

A basic assumption in setting up and applying leaf analysis standards, such as are presented in Tables I and II, is that they have rather general application in the range of soil and climatic conditions to which the crop is adapted. The validity of this assumption remains to be demonstrated conclusively, but a growing body of experience in various parts of the world gives encouragement that such standards do have rather broad application, and are indeed very useful, albeit not infallible, aids in solving practical problems in commercial fruit production.

INTERRELATIONSHIP OF ELEMENTS

Interpretation and application of experimental results would be much simplified if one could apply one mineral element to a plant and influence the nutrition of only that element. Unfortunately, such simplicity rarely, if ever, exists. An increase in one element influences the uptake and distribution of many elements in the plant. Shear *et al.* (183, 184) stress the im-

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TABLE II

MARGINAL RANGES OF NUTRIENT ELEMENTS IN TUNG LEAVES*

Element	Dry Matter Basis	Marginal Range
Nitrogen	%	2.0 to 2.3
Phosphorus	%	0.10 to 0.12
Potassium	%	0.80 to 1.00
Calcium	%	1.00 to 1.40
Magnesium	%	0.18 to 0.30
Zinc	p.p.m.	23 to 29
Manganese	p.p.m.	27 to 55
Copper	p.p.m.	4.1 to 5.1
Iron	p.p.m.	35 to 47

* Adapted from Drosdoff (71). The marginal range of an element is that below which the tree will respond, in increased growth and yield of fruit or both, to an application of the element, and above which a response is unlikely. The marginal ranges given for all the elements represent a satisfactory nutrient element balance for tung under orchard conditions.

portance of nutrient balance. Their data, concerned with tung trees, emphasize that the level of one nutrient element cannot be considered alone in interpreting plant responses. The relative level of other elements must also be considered. The studies by Cain (36 to 39) of deciduous fruits point out that the concentrations and ratios of elements in leaves do not necessarily reflect concentrations and ratios in other parts of the plant, nor do they indicate total absorption or uptake by the plant. In field experiments and in the diagnosis of nutritional problems in tree crops, it is seldom possible to destroy the whole plant and determine total uptake and concentrations of elements in all parts of the plant. As indicated in another section, studies of concentrations of elements in the leaves have been widely and successfully used in the diagnosis of nutritional problems and the interpretation of responses obtained in orchard fertilizer experiments. Some of the more common effects of increasing the concentration of one element on the level of other elements in leaves of fruit trees are described in the following paragraphs. In some instances, these interrelations are factors of considerable economic importance in orchard management.

One of the most common side effects of treatments which increase substantially the level of nitrogen nutrition in tree crops (as indicated by concentration in leaves) is a reduction in the level of phosphorus nutrition (11, 24, 170, 198). Indeed, experience in Florida indicates that it is possible to influence the level of phosphorus nutrition in citrus trees on sandy soil about as much by varying nitrogen fertilization as by varying phosphorus fertilization (198). In general, treatments or fertilizer applications which result in increasing the level of nitrogen nutrition are associated with an

increase in symptoms of potassium, boron, zinc, and copper deficiencies (11, 24, 32, 43, 71, 135, 198, 243). In many of these instances, data are available showing that these effects of nitrogen are accompanied by a decrease in concentration of the above elements in leaves. There are indications that an increase in nitrogen is associated with a decrease in chloride concentration and incidence of "tipburn" in avocado leaves (135). Chloride toxicity is at least one cause of "tipburn" in avocado.

Considerable evidence is accumulating that heavy phosphorus fertilization of orchard soils influences the uptake of the heavy metal group of micronutrient salts (14, 124, 163, 164, 198). For example, an increase in phosphorus has been noted, under certain conditions, to be associated with an increase in symptoms of zinc, copper, and iron deficiency (6, 88, 163, 172, 245). However, in an experiment on acid, sandy soil in a Florida citrus orchard, increasing levels of phosphate fertilization were noted to be associated with decreased copper, but with increased zinc and manganese concentration in the foliage (164).

Another very common instance of the interrelation of elements, found in both laboratory and field experiments with tree fruits, is the strong repressive effects that increasing the level of potassium nutrition has on the level of magnesium nutrition (13, 20, 21, 22, 72, 170, 198, 207). It is now known that heavy applications of potassium fertilizers, associated with a sharp increase in potassium concentrations in leaves, are likely to induce severe magnesium deficiency in fruit trees in most soil situations.

Even the heavy metal groups of micronutrient salts show some striking antagonistic effects. Studies in Florida have shown that toxic accumulations of copper in the soil interfere with the uptake of iron by citrus trees, and can induce severe iron deficiency symptoms (172). Additional studies have shown that very high levels of copper, zinc, and manganese applied in liquid, sand, or vermiculite cultures show some striking antagonistic effects between pairs on concentration in leaves, which were not always associated with similar relationships in rootlets (197, 203).

An interesting example of the interrelation of elements to symptom expression is provided by sand culture and field data with tung, showing that toxic symptoms associated with high boron in leaves were markedly reduced simply by increasing the magnesium level in leaves (141).

Another interesting example of an interaction of this type on plant response is provided by the work of Shear (186). His data show that zinc, when present in leaves in concentrations far above that required for elimination of zinc deficiency symptoms, markedly reduces freezing injury to shoots on young tung trees at a high potassium level, but produces only a slight reduction in injury at a low potassium level.

The material reviewed in this section and in the sections on Tree Behavior, Leaf Analysis, and Root Environment all suggest that it would be desirable to intensify research on the nature and seat of the antagonistic

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effects among nutrients noted. Also, it would be highly desirable to obtain more specific information on the effects on plant growth and nutrition of subtoxic, toxic, and excess levels of the mineral nutrient elements in plant tissues. Much of the past and present emphasis of such research is on deficient and adequate levels of these elements.

Scions and Rootstocks

Most orchard fruit trees are man-made synthetic or composite plants composed of a top or "scion" of one species or variety of a particular genus, grafted or budded onto a stem and root system or "rootstock" of a different variety or species of the same genus. In some cases, top and stock may be of a different genus. For example, it is a common horticultural practice to graft or bud a fairly wide range of species and varieties of the genus *Citrus,* all evergreen subtropical plants, on *Poncirus trifoliata,* a deciduous, fairly hardy genus of the Rutaceae.

Extensive evidence obtained largely during the last three decades has shown that rootstock variety or species, especially vegetatively propagated clones, markedly influences such factors as vigor and size of the scion, precocity of fruiting, certain chemical and physical aspects of fruit quality, internal anatomy of leaves, carbon assimilation rate per unit leaf area, hardiness, dormancy and mineral and organic composition of the tissues (*7*, 8, 9, 15, 55, 70, 75, 93, 94, 97, 153, 193, 219, 222, 227, 242, 247).

With apples, roots of stocks which characteristically dwarf the scion have an appreciably higher proportion of nutrient storage tissue in relation to water and nutrient-conducting elements than do roots of invigorating stocks (*7*, 8, 9). So far, no similar anatomical studies have been made with other orchard trees.

As might be expected, not only does the kind of rootstock influence the growth and fruiting of the scion, but also the scion influences the gross morphology, internal anatomy, hardiness, and ion selectivity of the rootstock. For example, with apples and citrus, studies have shown that scion variety may influence type of branching of the root system formed, the color of its bark, the internal morphology of the tissues and, presumably, the mineral and organic nutrient composition of the roots (97, 216, 217, 221). In addition, studies with citrus have shown that while the rootstock has an important effect on mineral composition of leaves in the top, the scion variety itself may have a modifying influence on nutrient ion selectivity of the rootstock or upward translocation of ions (61). It has been shown with apples growing on soils low in available potassium that certain stocks produce tops having a low potassium concentration in leaves associated with symptoms of potassium deficiency. Other stocks growing in the same soil, with the same top variety, produce normal leaves with normal potassium content (242). Similarly, orange varieties are much more prone to produce pathological symptoms of boron deficiency on sour orange than

on rough lemon rootstock under conditions of low boron availability in the soil (200).

In sand culture studies, Elberta peach trees tolerated a moderate amount of salinity from chloride salts better on Shalil than on Lovell rootstocks (104). Similarly, various citrus rootstocks influence tolerance of the scion to chloride, boron, and copper toxicity as indicated by foliage symptoms or concentration of these elements in the leaves or both (61, 75, 172). A limited number of studies have shown that the mineral nutrient status of the substrate may diminish or enhance an effect of stock on scion, but not change it qualitatively (93, 230).

Recent studies (175) have shown that a disorder known as "lemon collapse," associated with a partial girdling of the trunk due to necrosis of phloem tissue in the region of the union of the rootstock with the scion, was associated with an abnormally high concentration of sodium in the bark of the roots. Further studies (176) demonstrated that mechanical girdling of citrus trees, by means of removing a strip of bark around the trunk, also caused an abnormally high concentration of sodium in the bark of roots, suggesting that root starvation caused the root bark tissue to lose some of its ability to exclude sodium ion selectively.

Studies with grafted orchard trees, then, demonstrate that inherent genetic differences in rootstock varieties and species in selectivity in ion accumulation are a factor of importance in determining adaptation to varying soil conditions. Different rootstocks exhibit a remarkably wide range in ability to forage in the soil for individual nutrient elements low in availability. Rootstocks also show an equally wide range in ability to tolerate, or minimize the uptake, of individual elements in soils, such as chloride and sodium ion, which may be present in toxic concentrations. Studies with grafted orchard trees indicate also that genetic differences in the top, or scion, may influence the selectivity in ion absorption or translocation in the rootstock. This complex interaction of scion and rootstock combination with mineral nutrient ion uptake and distribution in various tissues of orchard trees is a factor of importance in determining vegetative and fruiting responses of such combinations, and their adaptation to varying levels of availability of mineral ions in the soil. It is clear, however, that this interaction is only one aspect of the complex interrelation of scion and rootstock. It seems likely that other factors, such as the capacities of the conductive tissues and others not yet clearly identified, play a more dominant role. Possibly the interplay of both the quantity and kind of organic nutrients and growth regulating substances produced by stock and scion is more fundamentally involved than mineral nutrition in determining the vigor, gross morphology, and fruiting behavior of a scion-stock combination in orchard trees.

Root Environment

Because of their large size, fruit trees require soils which provide the

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root system with good anchorage, and a large, deep, well-aerated reservoir of soil moisture, in order to meet the rigorous requirements of productivity and longevity set by horticulturists who plant them in orchards. Thus, the roots of fruit trees are more extensive and probe deeper into the subsoil, than the roots of most annual crop plants. Also, being perennials, the roots are functional through all four seasons, and over a span of a great many years. As a result, the soil in which the root system of a fruit tree functions is subject to a much wider range or gradient in physical environment and mineral nutrient availability than is the substrate of most annual crop plants.

For example, Ford (86) found mature citrus trees to have significant amounts of rootlets at a depth of 17 ft. below the surface, and 26 ft. in horizontal distance from the trunk. In winter, roots in the subsoil are appreciably warmer than roots in the topsoil. In summer, the reverse is true. Similarly, the physical and chemical make-up of the soil usually varies markedly with depth. Thus a portion of a tree's root system may be in an acid, well-aerated topsoil, and a portion in a poorly aerated calcareous subsoil. In deep sandy soils in particular, applied fertilizer materials leach downward through a column of soil and active roots which have a fractionating action on the nutrients. The data presented in Table III suggest that such fractionation with depth may be marked under certain conditions, and that the root systems of such trees are subject to both direct and inverse mineral nutrient gradients with increasing depth, depending on solubility, exchange, fixation, and rate of uptake of individual elements by roots. To further complicate the picture, the density of roots may also vary greatly with depth (86).

Some recent studies have shown that fertilizer treatments can significantly influence the concentration and distribution of citrus roots in the soil profile (34, 87, 204). So far, no evidence is available which affords a basis for theorizing, concerning the possible mechanism of such effects of applied fertilizers on tree rooting.

An evaluation of data from extensive surveys of citrus leaf composition, in the major citrus growing areas of the United States (170), seems to indicate that under orchard conditions, potassium concentrations in leaves may vary through a wide range in spite of a high level of calcium in them. In sand or water culture studies, the range of potash concentration in leaves, obtainable with a high calcium concentration in the substrate, appears to be more restricted (199). This suggests the hypothesis that under field conditions, the repressive effect of calcium on the uptake of potassium by roots may be reduced when the high calcium level is confined to one portion of the root system, and the high potassium to another. This would be the case with deep-rooting, potash-fertilized, orchard trees growing in a soil profile with an acid reaction and low calcium availability in the topsoil, but free calcium carbonate and high calcium availability in the subsoil. Simi-

TABLE III

EFFECT OF DEPTH ON COMPOSITION OF ROOTLETS OF VALENCIA ORANGE TREES ON ROUGH LEMON STOCK*

Fertilizer treatment†	Depth of rootlets in soil in inches	Concentration in dry matter‡						
		N %	P %	K %	Ca %	Mg %	Cu p.p.m.	Zn p.p.m.
$N_1K_1Mg_1$	0– 10	1.22	0.20	0.46	0.37	0.64	241	1810
	11– 30	0.89	0.14	1.05	0.22	0.41	59	585
	31– 60	0.80	0.14	0.94	0.16	0.32	16	161
	61–108	1.06	0.20	0.81	0.15	0.44	29	369
$N_2K_1Mg_1$	0– 10	1.46	0.15	0.37	0.34	0.47	296	2448
	11– 30	0.97	0.09	0.61	0.19	0.41	112	1545
	31– 60	1.15	0.10	0.73	0.13	0.25	37	472
	61–108	0.97	0.13	0.54	0.20	0.47	75	257
$N_2K_2Mg_1$	0– 10	2.02	0.18	2.20	0.66	0.41	250	2108
	11– 30	1.42	0.12	2.93	0.32	0.19	66	508
	31– 60	1.25	0.10	3.15	0.22	0.11	30	255
	61–108	1.32	0.11	3.51	0.20	0.13	61	191
$N_2K_2Mg_2$	0– 10	1.55	0.16	2.06	0.39	0.44	248	1721
	11– 30	1.12	0.09	2.46	0.24	0.29	80	648
	31– 60	1.14	0.09	3.34	0.19	0.15	81	408
	61–108	1.43	0.12	5.05	0.20	0.26	32	675

* Data obtained by the senior author while employed by U. S. Dept. of Agriculture, Orlando, Florida, from orchard experiments previously described (166).

† The symbol N_1 indicates a low level of nitrogen fertilization, and N_2 a high level, etc.

‡ Corrected to SiO_2-free basis by deducting weight of acid insoluble ash from dry weight of sample. Each value is the mean of four determinations from each of four plots.

larly, gradients in availability of other nutrients in relation to soil depth may modify, under field conditions, the sharp antagonistic effects which are observed with other ion pairs in sand, or pot culture studies.

These root environment factors, brought into sharp focus by studies with tree crops, suggest that more research is needed concerning the effects on the mineral nutrition of plants of the physical and chemical gradients to which the root systems of plants are subjected when growing in the field, but not when growing experimentally in containers of mixed soil, or in sand cultures.

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PHYSIOLOGY OF THE FRESH-WATER ALGAE[1, 2, 3, 4]

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From the viewpoint of the student of algal physiology, the topics which are justifiably part of the field are so diverse as to preclude complete mastery. This has proved especially true during the past decade. The extensive literature contained in the special reviews on mineral nutrition [Ketchum (206)], pigments [Strain (388)], blue-green algae [Fogg (106)], and mass culture [Tamiya (404)] suggests the variety and abundance of investigations of physiological problems concerning algae. General acceptance of Kluyver's concept of biochemical unity has led many to employ algae, which appear comparatively simple to culture, for the study of mechanisms common to all plants. Consequently, much of the literature is not oriented toward a comprehensive understanding of the metabolism of algae but, rather, is aimed in the direction of some problem of general physiology for which the algae are the most useful subjects for experimentation. This has resulted in the accumulation of more data concerning algae than would otherwise be available, but at the same time it has often led to remarkable contradictions in methods and results which are less prominent in a more unified discipline such as bacteriology.

A number of reviews of general algal physiology and of special facets of algal metabolism has given increasing coherence to this field of microbiology. The earliest, by Oltmanns (291), covers the period up to 1923. Klebs (218) in 1928 also reviewed conditions for reproduction and growth. Much of the work in the following 20 years is included in the classic volumes of Fritsch (127, 128). Recently Myers (273) and Blinks (38) organized a major segment of the literature concerning physiology and bio-

[1] The survey of literature pertaining to this review was concluded in August, 1957.

[2] This review is part of work supported by the Rockefeller Foundation and the Office of Naval Research.

[3] Scientific article No. A-654 Contribution No. 2844 of the Maryland Agricultural Experiment Station (Department of Botany).

[4] The following abbreviations will be used: ATP, adenosine triphosphate; CMU, 3-(*p*-chlorophenyl)-1:1-dimethyl urea; DNA, deoxyribonucleic acid; DNP, dinitrophenol; EDTA, ethylenediaminetetraacetic acid; IAA, indoleacetic acid; IBA, indolebutyric acid; NAA, naphthaleneacetic acid; PAA, phenylacetic acid; PAB, *p*-aminobenzoic acid; PGA, phosphoglyceric acid; RNA, ribonucleic acid; TCA, trichloroacetic acid; UDP, uridine diphosphate; 2,4-D, 2,4-dichlorophenoxyacetic acid.

[5] The author wishes to acknowledge the encouragement and assistance given by Drs. Hugh G. Gauch and Ronald Bamford during the preparation of the manuscript. The author is also indebted to Drs. Donald Wetherell and Constantine Sorokin for their critical reviews and helpful suggestions.

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chemistry. These were followed by a small book by Fogg (104) dealing entirely with algal metabolism. Two other books have been compiled from the contributions of many authors. The volume edited by Burlew (55) covers algal growth with emphasis on mass culture and that edited by Fry & Peel (129) deals with autotrophic microorganisms. In additon to the special reviews mentioned above, the following topics have been given detailed discussion: sexuality [Lewin (252); Hartmann (161, 162); Raper (333)]; photosynthesis [Bassham & Calvin (30); Aronoff (25); Krauss (228)]; toxic fresh-water algae [Ingram & Prescott (184)]; the role of vitamin B_{12} [Ford & Hutner (110)]; and phototropism and phototaxis [Reinert (337)]. An annual bibliography of all the literature pertaining to algae was begun by Silva (367) in 1954.

Discussion of the special topics covered in these recent reviews will be kept to a minimum here. In order to limit this review, the literature deals largely with fresh-water algae. This is an artificial boundary crossed not only by different species of the same genus [Lewin (254)], but also by individual species with rather broad salinity requirements as was found by Braarud (43) for *Exuviella baltica* and *Amphidinium sp.* Even less exacting tolerances have been observed by George (140) in *Stichococcus bacillaris, Chlorella ovalis,* and *Chlorella stigmatophora* and, as was shown by Allen (16), euryhaline species of *Platymonas* exist over a range from fresh water to salt concentrations twice that of sea water. It will occasionally be helpful to add supplementary information about marine forms. The present review deals with the literature after 1950 except where pertinent earlier papers have been omitted by other reviewers.

Cultures Techniques and Growth

A variety of culture techniques is available for preparing algae for study and for maintaining them during experimentation. The responses of cultures to nutrients or other agents are commonly measured as growth *in situ,* but often algae are not subjected to experimentation until after they have been harvested or transferred to apparatus very different from that in which they were grown. Measurements can be made in such apparatus, as during studies of respiration, or the algae may be analyzed subsequently to determine the effects of a given treatment. A majority of physiological studies deals with unicellular or coenobial forms isolated in pure culture prior to experimentation. Pure cultures are commonly obtained from the large collections in the United States described by Starr (383) and in Europe from those of George (141) and Pringsheim (315). Isolation from nature has been simplified by the use of antibiotics, a method introduced by Provasoli *et al.* (330), Spencer (379), and Goldzweig-Shelubsky (149). Although Myers *et al.* (279) have shown that *Chlorella* can grow in open cultures under certain conditions without apparent inhibition by bacteria, and Pringsheim (314) has pointed out that pure cultures are neither always neces-

sary nor an end in themselves, there is no doubt that there is greater security in results obtained with uncontaminated cultures. In open cultures, control of the common contaminant *Bacillus subtilis* has been reported with 0.02 per cent β-naphthol or salicylic acid without damage to *Chlorella* [Wai (421)]. Gerloff *et al.* (143) have perfected a technique for purification of blue-green algae by irradiation with ultraviolet light. Allen (14) has pointed out the desirability of avoiding mutagenic agents in obtaining pure cultures and employs, instead, repeated transfers from the peripheries of spreading colonies. Kingsbury & Barghorn (217) have provided a method for preparing silica gel from the commercial preparation called Ludox. The silica gel may then be used to purify and culture algae on solid media free of any organic carbon source. Special culture techniques have been described for dinoflagellates by Sweeney (390) and Delany (77), for *Nitzschiae* by Wielding (444), and for brackish and marine forms by Provasoli *et al.* (327)

Studies of growth rates are generally carried out in test tubes or in flasks supplied with glass tubes through which a stream of CO_2-in-air may be bubbled. Test tubes are convenient for such studies since they can be periodically removed from an illuminated water bath and inserted into a spectrophotometer for measurement of optical density. Larger devices require frequent sampling and may demand mechanical agitation in addition to bubbling to prevent sedimentation [Krauss (225)]. Reisner & Thompson (340) have solved the requirement for large samples of cells by building culture-tubes with capacities of 2 l. When maximum growth is not required or where algae are growing on organic carbon sources, shaking devices such as those employed by Walker (422), Algeus (12), and Galloway (134) serve for the simultaneous culture of a large number of tubes or flasks. Deep-tank "fermentations" [Pruess *et al.* (331)] employing glucose and catalytic amounts of light have proved successful for a number of species of green algae where large amounts are desired. Gaevskaia (132, 133) conducted studies of the suitability of various types of vapor lamps for algal growth and reported deleterious effects of ultraviolet irradiation from mercury vapor fluorescent lamps. No device can be considered to maintain a maximum growth rate or exponential growth at any rate, without provisions for adequate nutrition, light, and temperature. In test tubes, or larger devices of the same basic pattern that are batch cultures, maximum growth will be exhibited only until mutual shading restricts light or until nutrients are exhausted or in disbalance. Photoelectric control of cell concentration and nutrient addition developed by Myers & Clark (276), and recently adapted to a smaller scale by Phillips & Myers (302), is the classic solution to this problem. A regimen of daily harvesting and replacement of nutrients has provided constant yields for periods of several months in mass cultures of *Chlorella* and *Scenedesmus* (230).

Continuous culture has occupied the ingenuity of a number of micro-

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biologists [Golle (150); Finn & Wilson (100)]. Monod (271) developed the theory of continuous culture and Novick (288) has recently compared the devices built to exploit the concept. Of these, the chemostat and bactogen appear to be useful for growing algae for physiological study. However, an essential feature of such apparatus is that the growth of the organism be limited by a suboptimum level of some component of the medium. Growth is kept at an exponential, but restricted, rate by a constant drip that adds a specific increment of new medium containing the limiting factor at measured intervals. Steady periodic addition of complete medium to optimally-illuminated cultures of algae in such apparatus may fail to compensate for minor fluctuations in the growth rate and dilutions become too rapid or too slow to maintain constant populations. Even photoelectrically controlled populations (276, 302) will, in time, alter the medium unless the elements therein are quantitatively matched to the absorption of the algae. It has been considered important to use, as an experimental standard, cells which are at their maximum synthetic capacity. Any, even brief, limitations of a factor required for growth may result in abnormalities which are difficult to interpret.

The problem of producing algae in a uniform, metabolic condition has been given new perspective by the work of Tamiya *et al.* (406, 407) on cell synchronization. Their observation that *Chlorella* cells were different in average size and chemical composition during alternating periods of light and dark led to a description of the life cycle. Mature cells grown in the light, called "light cells," will divide in darkness to give young daughter cells, called "dark cells." A population of cells after a dark period, or a continuous period of low light intensity, therefore, should contain a much greater percentage of small young cells than a randomly-growing culture under continuous light of high intensity. Partially mature "light cells" will not divide in darkness, and, by giving several cycles of alternating 12 hr. light and 12 hr. dark, the life cycles of almost all of the cells will become synchronized and they will begin growth in the next light period as "dark cells." Actually the terms "light cells" and "dark cells" refer only descriptively to stages in a continuing process of aging or development. The metabolism of the two types of cells and of their intermediate stages is remarkably different. The photosynthetic quotient is equal to one for dark cells but is as much as three for light cells [Nihei *et al.* (287)]. Furthermore, the rate of O_2 evolution of light cells is only one-fifth that of dark cells. The activity of dark CO_2 fixation diminishes during the transition from dark to light cells, and light-limited photosynthesis and chlorophyll content also decrease during this period. The relative quantum efficiency in light cells is only one-half to one-third that of dark cells. Hase *et al.* (166) examined N, P, K, Mg, and S levels as per cent of dry weight and found that, while most elements varied slightly during a life cycle, S concentration was sharply reduced prior to cell division but rapidly increased at cell

division. Nihei *et al.* (287) demonstrated a rising respiration rate for *Chlorella ellipsoidea* with the maximum in mature light cells. This has been contradicted by Sorokin & Myers (378) who showed that in *Chlorella pyrenoidosa,* strain 7–11–05, after a sudden rise, respiration declines as the cells mature. Photosynthetic studies by Sorokin (374) during the life cycle of strain 7–11–05 show a rapid rise in photosynthesis by the third or fourth hour and then a decline in photosynthesis continuing to maturity. This rapid rise was not observed by Nihei *et al.* in *Chlorella* or by Neeb (281) in *Hydrodictyon.* Interpretation is complicated by work that revealed a photochemical liberation of O_2 accompanying phosphorylation during nuclear division [Nihei (286)]. Iwamura (185) and Iwamura *et al.* (186) studied the changes in DNA:RNA ratios in *Chlorella* and observed that prior to mitosis, DNA dropped markedly. This further indicated a fundamental shift in metabolism at the onset of cell division.

The dramatic differences in cells of different ages cannot be detected in perfectly randomized cultures, but variations in potentially limiting factors such as light, temperature, or nutrients may reduce randomness and accidentally induce some degree of synchronization. Such an effect would not only be present when a given factor was controlling synchronization, but could be present for many generations thereafter. A shift in age composition may be one cause for the variations in the performances of cells with different past histories. The difficulty in determining the condition of the cells, even in exponential growth, has been emphasized by Fogg (107) who observed that the shift from protein to fat or carbohydrate synthesis which is expected in N-deficient cells occurs long before exponential growth ceases. Manipulation of cells prior to experimentation may also directly alter their physiology, though algae are often considered insensitive to rigorous preparative procedures. *Nostoc muscorum* centrifuged at 23,000 g and *Chlorella pyrenoidosa* at 145,000 g for 2 hr. in a refrigerated centrifuge showed no loss in their subsequent photosynthetic capacity [Clendenning & Brown (61)]. Nevertheless, other results can be expected when larger algae are centrifuged, as in the case of *Nitella flexilis* [Breckheimer-Beyrich (46)].

Of special concern are criteria for determining growth rates. Techniques most frequently used are: cell count, dry weight, and photometric measurement. Cell counts give only cell number. For determinations of cell number, Gaevskaia (131) has employed an automatic cell counter much like those used for blood cell counts. Dry weights, on the other hand, show large deviations when samples are small. In practice, many investigators use the colorimetric method in which it must be assumed that, at the wavelength selected, there is equivalence between concentration and optical density. Inasmuch as Beer's Law is likely to be valid only for true solutions, it is surprising that it holds for suspensions of algae. In tubes containing *Nitzschia closterium,* Spencer (380) has reported loss in light transmission

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due to scatter and he has modified his colorimeter by providing a small opening for incident light and a large opening for transmitted light on opposite sides of the cuvette. Certain colorimeters incorporate this featuure and are much superior to others. The most accurate method of measurement for small particles in suspension is the nephelometer which essentially measures light scatter. Such turbidity measurements were shown by Diller & Kersten (78) to be closely correlated with dry weight.

Attention has been given to mathematical aspects of growth kinetics. Myers (274) has adapted the fundamental concepts of microbial growth to the typical autotrophic characteristics of *C. pyrenoidosa*. Tamiya (402, 403) and Tamiya *et al.* (405) have endeavored to derive equations that would make possible the prediction of yields if all factors of the environment are known and appropriately weighted as to their effect on growth. They also compensate for the relative concentrations of "light" and "dark cells" in the culture. Within limits, the equations are surprisingly useful. Variations depending on configuration of vessels and degrees of agitation are very difficult to integrate and it will not be easy to set up satisfactory equations for all conditions. Other efforts toward the formulation of over-all mathematical theory for growth have been made [Golle (150); Finn & Wilson (100)]. Odum & Pinkerton (290) have speculated that for maximum power output in biological systems the efficiency must be low—ordinarily under 50 per cent. Their interpretation of the over-all power efficiency equation and entropy, as time's speed-regulator, indicates that efforts to increase the efficiency of algal photosynthesis and growth are likely to be fruitless.

The maximum sustained growth rate of Emerson's strain of *Chlorella*, near 3.0 $\log_2$ units per day, has long been accepted as typical of the most rapid obtainable. There is now considerable interest in a strain of *Chlorella* isolated by Sorokin & Myers (376, 377), designated 7–11–05, which has a rate of 9.2. Kratz & Myers (223) have also studied a blue-green alga, *Anacystis nidulans* (probably more accurately a *Synechococcus*), that is even more prolific with an optimum rate of 12.0. Rapid photosynthetic and growth rates in these algae may best be attributed to a high temperature-tolerance which permits undamaged and accelerated enzyme activity. At 25°C. the rates for these algae are no greater than for the more familiar strains, but at 39°C. the difference is most conspicuous. The rates for strains of *Chlorella, Scenedesmus,* and *Chlamydomonas* have been compared at light intensities up to 10,000 ft.c. by Sorokin & Krauss (375). The high temperature strain of *Chlorella* is inhibited by intensities above 1000 ft.c. when grown at 25°C., but not below 3000 ft.c. at 39°C. The high temperature apparently permits a higher absorption of light energy without damage.

There have been numerous attempts to explain the failure of natural populations to grow as rapidly as laboratory cultures. Even during periods of algal "bloom," high rates are either not achieved or are of inexplicably short duration. Although Gerloff & Skoog (144) and Tucker (417) have

reported exceptions, nutrient deficiency is probably the most general cause for suboptimum growth in nature. Other factors of the environment also may control growth. Talling (400) has attributed the drop in growth rates of *Asterionella, Fragillaria,* and *Tabellaria,* from two in the laboratory to one or less in cultures supplemented with nutrients and immersed in natural waters, to be due to suboptimum light and temperature.

Inorganic Nutrition

The requirement for media suitable for growth has been the traditional stimulus for studies of inorganic nutrition. Countless media have been proposed for the culture of various species. A useful table giving the composition of six of the more common media are given in the handbook edited by Albritton (3). Data are also included indicating the essentiality of the elements. Provasoli *et al.* (327) have written a detailed account of the composition and preparation of media for brackish and marine algae. Pirson (304) has discussed the roles of the elements in green plants and has given major emphasis to the algae. Ketchum (206) and Krauss (226, 227) have also reviewed the field of inorganic nutrition of algae. Consequently, only topics of current interest will be covered here.

The elements now considered to be inorganic requirements for all algae are N, P, K, Mg, Fe, Ca, Zn, Mn, and Cu. A number of other elements have been identified as requirements for certain species or groups of algae. To the list of major nutrients, silicon should be added for diatoms, and sodium has been shown by Allen (17) to be essential for *Anabaena cylindrica.* Also sodium appears to replace K in species of 12 other genera of blue-green algae [Allen (14); Kratz & Myers (223)]. Both sodium and potassium should be included in media for blue-green algae of unknown nutritional requirements. Walker (422) has reported the complete substitution of strontium for Ca in *Chlorella,* and Kellner (205) has achieved partial replacement, and then complete mutative adaption, of *Ankistrodesmus* to rubidium in the absence of K. This substantiates the early work of Pirson (303) who grew dense cultures of *Chlorella* in the complete absence of K with rubidium as a substitute. Holm-Hansen *et al.* (173) have established cobalt as a micronutrient for *N. muscorum, Calothrix parietina, Coccochloris peniocystis,* and *Diplocystis aeruginosa.* Levels as low as 2 p.p.t. were sufficient to differentiate from controls and 400 p.p.t. was optimum. The niche for cobalt in vitamin B_{12}-synthesizing Cyanophyceae is obvious, but the stimulatory effect of cobalt on the growth of green algae (227) suggests that it may also play a role in groups other than the blue-greens, perhaps in an indirect protective role as suggested by Galston & Siegel (135). Holm-Hansen *et al.* found no response to added molybdenum, but Arnon *et al.* (21) showed that Mo was essential for nitrate reduction in *Scenedesmus.* Reductions in photosynthesis, respiration, and chlorophyll content were also noted by Loneragen & Arnon (257). Wolfe (453, 454) examined the role of

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Mo during nitrate reduction in *A. cylindrica* and has made the surprising observation that, in the absence of Mo, NO_3 is reduced to NH_3 but is not further synthesized to protein. These studies support the early work of Bortels (42) on the requirement for Mo in the N-fixing Nostocaceae. Vanadium, a common contaminant of ferric chloride, has been demonstrated as a micronutrient for *Scenedesmus* by Arnon & Wessel (22). Vanadium has been demonstrated subsequently by Warburg *et al.* (429) to be associated with the reduction of CO_2. Boron has routinely been included in media without substantial support for its essentiality, except in the reports by Eyster (95) that boron is required for *N. muscorum,* and those showing stimulation for *Chlorella* [Geigel (136)], diatoms [Herzinger (172)], and *Ulva* [Suneson (389)]. Recently McIlrath & Skok (262) have shown a 137 per cent increase in dry weight of *Chlorella vulgaris* over boron-free cultures after 24 days in a medium containing 0.5 p.p.m. boron. Contrary evidence indicating that boron is not an essential element was presented earlier by Stegmann (384). Critical examination of the boron requirements in algae is long overdue. Hervey (171) has observed a stimulatory effect of sublethal concentrations of chromium on the growth of several species of unicellular algae. However, the long period over which growth was observed (15 to 56 days) and the poor environmental conditions lead one to suspect secondary effects not directly connected with nutrition. Positive essentiality is best established in terms of the effect of the presence and absence of the element on the optimum growth rate. Quantitative analyses of large scale cultures growing autotrophically at a constant rate for many weeks have yielded extensive data on the inorganic content of *Scenedesmus* and *Chlorella* (230, 233). With periodic addition of the essential elements to match daily harvests of the algae, balanced replacement formulae for major and micronutrients based on these analyses have made possible a continuous culture (230). In other analytical studies, Knauss & Porter (219) showed that *C. pyrenoidosa* accumulated isotopes of P, Ca, S, Fe, Mn, Zn, Cu, and Sr in excess of immediate requirements. Absorption of all but P and S were in direct proportion to their concentration in the medium, and P and S were absorbed at a constant level only at high concentrations. Such data emphasize the capacity of the algae for luxury consumption of inorganic elements.

Walker (424, 425), in a series of experiments, with *Chlorella* growing photoheterotrophically, has determined the micronutrient sufficiency to be 30 μg. Fe, 2.5 μg. Mn, 4.5 μg. Zn, and 10 μg. Ca per gram dry weight of algae. Strontium competitively inhibits Ca uptake. Though Sr can replace Ca without damage to *Chlorella,* both *Scenedesmus obliquus* and *Selenastrum minuta* cannot utilize Sr and *Coccomyxa pringsheimii* is inhibited by its presence. Rice (343) has determined a differential uptake of strontium and yttrium by 12 planktonic algae showing various degrees of selectivity, from *Carteria* which absorbs strontium and excludes yttrium to *Gyrodinium* which

does the opposite. Spooner (382) has also recorded the absorption of strontium and yttrium in marine algae. The reasons for these differences are difficult to understand and should offer a fruitful field of investigation. In dealing with beta-emitting isotopes, however, secondary effects, which may confound the experiment, can be expected. Tritium oxide is especially damaging to cell division in *Chlorella* [Porter & Knauss (309); Porter & Watson (310); Weinburger & Porter (440)], and even deuterium oxide alters the photosynthetic rate [Horwitz (179)].

The assimilation and function of individual elements have been examined. Phosphorous assimilation by P deficient cells of *Chlorella* was measured by Al Kholy (13) who found that growth continued until the P content dropped to 1×10^{-7} μg. per cell. Goldberg *et al.* (148) observed bound and unbound P fractions in *Asterionella japonica*. The minimal PO_4, 5×10^{-8} μg. atoms/cell, was the water-insoluble fraction. Mackereth (260) determined that *Asterionella* can take up and store P from a concentration of less than 1 p.p.m. The limiting requirement is 0.06 μg. P/10^6 cells, so 1 μg. P can produce 16×10^6 cells before limitation sets in. Gerloff & Skoog (144) have also studied luxury consumption of P and N in *Microcystis aeruginosa* and have concluded that these elements were not limiting in the lake waters where blooms were common. Algae are capable of absorbing organic as well as inorganic phosphorous compounds. Harvey (164) has now demonstrated that inositol-hexaphosphate and glycerophosphate are absorbed by *Nitzschia closterium* in both light and darkness, but Rice (341), in a study of P exchange, showed that little conversion of inorganic P to organic compounds occurred in the dark. Nihei (286) reported a phosphorylative process in the absence of CO_2 with synchronized cells just prior to division. For each mole of PO_4 absorbed, 3.4 moles of O_2 were evolved. The PO_4 was fixed as metaphosphate. This appears to be a direct photochemical reduction and synthesis of energy-rich metaphosphate as an alternative to CO_2 reduction. Schwinck (361) also found the highest activity of inorganic P^{32} absorbed by *Ankistrodesmus* to be in the ATP of an ethanol extract of cells which had been actively photosynthesizing. The $-\Delta F°$ for inorganic pyrophosphate has been calculated as 8000 cal. by Meyerhof & Oesper (265). Polyphosphates are of wide distribution in the algae and could serve both as a phosphate reservoir and an effective energy store, as suggested by Stich (386) and Thilo *et al.* (410) for *Acetabularia*. If this is true, it is difficult to understand why P deficiency is much more damaging to both heterotrophic and autotrophic growth, than to mixotrophic cultures of *C. vulgaris* as reported by Bergmann (36). A large-ring polymer of phosphate containing 50 units has been postulated by Yoshida (465) based on work with yeasts where the majority of phosphate in the cells was in the form of a large polymer soluble in hot 5 per cent TCA but not in cold 5 per cent TCA as are the 10-unit chains. Unfortunately no studies have yet explored the structure of the metaphosphate (preferably termed polyphosphate) in algae.

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Studies by Wassink *et al.* (434) on the purple sulfur bacterium, *Chromatium,* for which evidence was presented that energy-rich phosphate bonds were formed in the light, have been followed by confirmatory evidence for *Chlorella* (431). This work has been expanded by Wintermans & Tjia (450), Wintermans (449), and Wassink & Rombach (433). Wintermans (448) has also supported his suggestion that phosphate bond energy is first generated in light by a reoxidation of a primary photoreductant coupled to ATP formation and then transferred to polyphosphate especially in the absence of CO_2. A series of experiments by Kandler (197, 201) have also focused attention on the connection between photosynthesis and the phosphate economy of algae. In his early work, Kandler measured a sudden drop in the inorganic phosphate level during the first 30 sec. after illumination of *Chlorella pyrenoidosa* kept previously in the dark. This was followed by an increase when the cells were returned to the dark. The level of inorganic P was the same under constant light or darkness. He postulated that part of the light energy is directly transformed into ATP and that the ATP could play a role in assimilation of CO_2 as well as other energy-requiring processes in the cell. Basing his experiments on the observation that 0.1*M* cyanide inhibits oxidative phosphorylation but not dephosphorylation, he was able to show that in the light P was retained in the cell despite the presence of cyanide. In the dark with glucose, P is liberated. Further demonstrations of the dependence of glucose uptake on photophosphorylation were the increased glucose uptake of starved cells when illuminated and the failure of anaerobic conditions to block glucose uptake in the light. Additional support for ATP production in the light, and subsequent availability for glucose assimilation, was obtained in experiments showing specific respiratory inhibitors to be less effective in the light.

Sulfur metabolism has been studied by Shrift (364, 365) especially as it is affected by selenium poisoning. *C. vulgaris* is able to use either D or L-methionine as a S source. Competitive antagonism has been established between L-methionine and seleno-methionine which may act at two sites—one in the membrane and the other inside the cell. *Chlorella* appears to build up resistance to Se toxicity possibly through the production of an excess of reduced S compounds.

The close relationship between Mn and photosynthesis has been established by Pirson & Bergmann (305) who reported that *C. vulgaris* grew heterotrophically on glucose in the absence of Mn, but that Mn was required for autotrophic growth. In view of the frequent indentification of Mn as an enzyme activator, it appeared surprising that it could be eliminated from all but the photosynthetic reaction. Reisner & Thompson (339) have contradicted these findings, suggesting that Mn may have been a contaminant in the glucose. Their experiments do show, however, two times as much Mn in cells at the cessation of growth in cells grown autotrophically in media deficient in Mn as in those grown heterotrophically. Eyster *et al.* (96)

showed a 25 per cent reduction in photosynthesis and a failure of the Hill reaction in Mn-deficient cells. Kessler (210) also showed that there is a 25 per cent reduction of photosynthesis under Mn deficiency, but that photoreduction is not inhibited in these algae when adapted to H reduction. Manganese is known to play a role in the reduction of nitrates by *C. vulgaris*. This is supported by the observation of Kylin (237) that Mn stimulates the growth of *Ulva* sporlings if NO_3 but not NH_3 is the N source. Pringsheim (317) demonstrated that the normal shape and brown color of loricas of *Trachelomonas* depend on Mn in the culture medium. Singh's (368) failure to find coloring of loricas in cultures where Mn was present does not detract from the importance of Mn in normal lorica formation. In quantitizing the requirement, Harvey (163) found that 0.5 to 2.0 μg. Mn/l. allowed vigorous growth of *Chlamydomonas, Chlorella,* and members of the Cryptomonadaceae and Chrysomonadaceae. He observed that Mn was rapidly exhausted from media by *Chlorella.* Gusseva (157) reports that the Mn requirement of algae is high if the Fe requirement is also high. Pollard & Smith (308) reported heavy adsorption of Mn by algae polysaccharides.

The composition of the frustule has made obvious the requirement for silicon in diatoms. In a study of the sources and forms of Si utilized by diatoms, Lewin (246) showed that 35 mg. Si/l. is optimum for dense cultures of *Navicula pelliculosa,* and that only compounds yielding soluble Si (orthosilicic acid) were capable of supporting growth. Agar ash can be expected to supply 2.4 mg. of soluble Si/gm. dry weight of agar, and intact agar may release 1.5 mg./gm. for the support of growth. Patrick & Wallace (300) have also reported a stimulatory constituent in agar ash. Apparently there is an unknown organic stimulant in agar as well, but this may be a manifestation of physical dispersion. Lewin (247) has reported variations of SiO_2 in *Navicula* between 4 and 22 per cent of the dry weight. Cells in the exponential phase contained the least silicon. Jørgensen (194) has conducted experiments which indicate that a lower Si level can be expected as growth continues, probably because of the release of an inhibitor to Si uptake. It is not clear whether this phenomenon is the result of inhibitors, or whether the composition of cells varies as their rates of growth or syntheses change. The minimum level for Si in the medium was 30 to 40 μg. Si/l. below which diatom growth was much reduced (193). Jørgensen (195) has also demonstrated that the Si in the frustules of *Nitzschia* and *Thalassiosira* is dissolved even at a high pH. He postulated that Si was complexed in a slowly-soluble compound similar to the Si-galactose complex in cereal straw. Lewin (245) identified a polyuronide of glucuronic acid residues which surrounds the cells of *Navicula* after they have stopped dividing. This may serve to protect against Si loss. The mechanism of Si uptake has also been explored and evidence points to a close link to aerobic respiration [Lewin (244)] and to sulfhydryl groups in the cell membrane [Lewin (243)].

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Magnesium at and below concentrations of 2.8 p.p.m. has been shown to be insufficient for prolonged normal growth of *Chlorella* cultured on glucose in the light. Finkle & Appleman (99) found that, after cell division stopped in Mg-deficient cultures, increases in cell volume continued to 20 times the normal volume. Chlorophyll was proportional to Mg from 0.2 to 2.8 p.p.m. and catalase activity was also reduced in the low Mg cultures. Less Mg appeared to be required for catalase activity than for chlorophyll synthesis (98). Halldal (158) has also discussed the relationship of Mg to adenosinetriphosphatase activity in causing positive phototaxis in *Platymonas*. The action of Mg is opposite that of calcium. However, Provasoli *et al.* (328) investigated the effect of the Ca/Mg ratios on the growth of marine flagellates and found that normal growth occurs under widely varying ratios as long as the monovalent/divalent ratio and the concentration of the medium are near optimum for growth.

It has long been known that the presence of the correct elements in a nutrient solution does not guarantee that they will be available to the algae. This is especially true of the micronutrients or "trace metals" which readily form highly insoluble oxides or hydroxides. Since the papers of Schatz & Hutner (353) and Hutner *et al.* (183), various chelating agents [Chaberek & Bersworth (56)]—especially EDTA (57)—have been used routinely in culture media. Waris (430) has shown that the pH range for desmids is widened by EDTA, indicating that pH acts partially through its effect on the solubility of nutrients. Spencer (381) reported that the toxicity and growth promotion of EDTA complexes of Cu and Mn are related to the ionic concentrations of the metals and not to the total chelate content. He further pointed out that EDTA was stable and not metabolized. Other data from experiments with ^{14}C-labeled EDTA show that it is absorbed and broken down, and that the algae secure the metals from the chelates in this manner (229, 230). This is also supported by observations on higher plants. A mechanism for chelate breakdown either in the cells or in the medium is suggested by the work of Krugers & Agterdenbos (236) and Jones & Long (192), who have established that Fe · EDTA chelates decompose rapidly in the light. The breakdown of the complex would make the metals progressively available to the algae. In sharp contrast to most of the data concerning unavailability of the trace metals in colloidal micelles, Goldberg (147) has reported that *Asterionella* uses only particulate or colloidal iron as a nutrient and that ferric iron as citrate, tartarate, or humate was not available for growth.

Nitrogen Metabolism

Nitrogen fixation, demonstrated among the algae only in the Nostocaceae, Oscillatoriaceae, Scytonemataceae, Stigonemataceae, and Rivulariaceae (102, 446) has been studied from economic as well as scientific points of view. In a series of experiments primarily with *Tolypothrix tenuis*, Watanabe (436) showed that, in four years after inoculation with algae, fields of

rice yielded 128 per cent more than the uninoculated controls. The plants in the inoculated paddies contained 7.5 lb. more N per acre than the controls (437). De & Mandal (76) also were able to obtain from 13 to 44 lb. of fixed N per acre from unfertilized, waterlogged rice-soils. In laboratory cultures, Fogg (102) found that *Mastigocladus laminosus* can fix 12.88 mg. N/l. in 20 days. Nitrogen fixation in soils appears to be surprisingly efficient. Singh (369) reported the reclamation of alkali lands in India by permitting blue-green algae to grow in temporary paddies created during the rainy season. The algal- and lichen-stabilized crusts of desert soils in the United States also serve as a reservoir of fixed nitrogen (363).

Atmospheric nitrogen, however, is not generally as efficient as a source for growth as NH_3 or nitrate. Kratz & Myers (223) showed that N fixation in *Nostoc* supported only 75 per cent of the growth obtained on nitrate. In contrast, Allen & Arnon (18) compared the rate of growth of *A. cylindrica* on N_2 and nitrate. The rate was as high and, in fact, higher on N_2 as long as sufficient Ca was present in the medium. A puzzling phenomenon is the excretion of nitrogenous compounds into the medium by N-fixing algae. Henriksson (169) noted that a strain of *Nostoc* isolated from the lichen, *Collema,* secreted one-fourth of the fixed N into the medium during a three-week period of growth. Watanabe (435) examined species of *Tolypothrix, Calothrix, Anabaenopsis* and *Nostoc* and found that all lost nitrogen. *Calothrix* excreted the most, 2.2 mg. of the 5.5 mg. N/100 ml. of culture which it had fixed. This N was primarily in aspartic and glutamic acids and alanine. It is not clear what the survival value of excretion of N may be to algae obliged to reduce atmospheric N to meet their needs. The amino acid products of assimilation are similar regardless of the source of N, and methionine is conspicuously low in the nine blue-green species examined by Williams & Burris (446) regardless of whether they fixed nitrogen or not.

Sources of N in media have been widely tested. Algeus studied the use by green algae of glycine (5), alanine (11), aspartic acid, succinamide, asparagine (7, 8), and glutamine (10). Only alanine fails as a N source for *Scenedesmus* (6). Amido-N is assimilated more readily than amino-nitrogen. It appears that aspartic acid does not hold the key position in amino acid synthesis which might have been expected from studies with higher plants. In *Chlorella,* Arnow *et al.* (23) found that only arginine, citrulline, ornithine, and urea served as N sources, confirming the operation of the Krebs-Hensleit cycle demonstrated earlier by Walker & Myers (427). Arginine utilization is activated by Mn, but the metal is not necessary for citrulline assimilation. In *Nostoc,* Linko *et al.* (255) determined that although citrulline was heavily labeled after 5 min. of photosynthesis with $^{14}CO_2$, free, labeled urea or arginine were not found and, thus, the absence of the cycle in this organism was indicated. Early studies with urea had indicated that it does not serve as a direct C source, but Ellner & Steers (86) showed that not only does urea act as a N source but also as a C source with 25 per cent

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of the guanine C in *Chlorella* and 100 per cent in *Scenedesmus* coming from urea. This holds even though growth will not take place in the absence of carbon dioxide. This is true despite the capacity of *N. muscorum* to hydrolyze urea and oxidize it to the carbonate from which CO_2 can be absorbed in the usual manner [Allison *et al.* (20)]. Sager & Granick (351) have also shown urea as well as glutamic acid assimilation in *Chlamydomonas reinhardti* in either the light or the dark. The absence of urease in *Chlorella ellipsoidea* supplied NO_3, urea, arginine, citrulline, or ornithine [Hattori (167)] is difficult to explain. Requirements for specific amino acids have been frequently found among the Euglenophyta and Chrysophyta [Hutner *et al.* (181)] and Storm & Hutner (387) have shown a nucleic acid requirement for *Peranema tricophorum* which is satisfied by uracil and guanylic, cytidylic, and adenylic acids.

Preferential absorption of NO_3 over NH_3 has been established in *Haematococcus* and *Pandorina* although some NH_3 was concomitantly absorbed [Proctor (322)]. In *Chlamydomonas* the reverse is true and NH_3 is absorbed almost exclusively as is true also with *Chlorella vulgaris* [Schuler *et al.* (360)]. *Gonium, Chlorella,* and *Scenedesmus* evidenced a less conspicuous preference for ammonium-nitrogen. Also Schröder (359) has shown that the cells of *Stichococcus* grown on NH_3 are more frequently bent than when grown on NO_3. Details of the assimilation of NH_3 and NO_3 by *C. vulgaris* have been studied by Syrett. When NH_3 is added to N-starved cells, there is an immediate increase in respiration. The NH_3-stimulated respiration is cyanide-sensitive and has an R.Q. of 0.75 compared to 1.2 for normal respiration. Glucose does not accelerate either NH_3 assimilation or respiration rates in N-starved cells (393). There is a rapid assimilation of NH_3 nitrogen into soluble N, especially amide and free amino-nitrogen. Much less soluble N is found when NO_3 is absorbed (396). The pool of soluble N drops in glucose-sufficient cells as protein is formed. Nonreducing sugars and acid-hydrolyzable polysaccharides are also metabolized rapidly during this period (394). Basic amino acids, however, formed an abnormally high percentage of the free amino acids even after 24 hr. (399). The total amount of O_2 production is the same with NH_3 or NO_3, but there is a greater volume of CO_2 evolved with NO_3 or nitrite. This extra CO_2 is the result of the requirement for reducing H which must come from respiration (395). When hydroxylamine is used as the N source, the gas exchange is entirely different from that expected if it were assimilated through the same intermediates as the other N sources. All data suggest a close link between respiration, NO_3 reduction, and N assimilation (397). In the dark the assimilation of NH_3 nitrogen by N-starved cells is accompanied by considerable C fixation (398). There must be a different balance in the reaction cycle in the NH_3- and NO_3-absorbing cells. It is possible that the reason the growth rates of the algae are the same in NO_3 and NH_3 media is that there is a balance among the Krebs Cycle acids that

acts as a governor to growth regardless of the N source. This view is supported by the evidence of Bongers (41) that the limiting factor for N assimilation is the availability of suitable C skeletons. Bongers further showed that there is a strong secretion of NO_2 and NH_3 when conditions for absorption are favorable but when the organic C supply is low.

The pathway for the reduction of NO_3 is through NO_2 and hydroxylamine to ammonia. Nitrate and nitrite reductase have been identified in *Scenedesmus* [Omura (292)]. Nitrate reduction to NO_2 is accelerated in the light indirectly through the production of photosynthate, but NO_2 appears to be reduced directly by light [Kessler (211); Van Niel *et al.* (419)], or by a transitional reducing agent [Harvey (165)]. In the light in the absence of CO_2 *Chlorella* may produce O_2 by reducing NO_3 and photosynthesizing the respiration-released CO_2 [Davis (75)]. In the dark NO_3 reduction is coupled with aerobic respiration [Kessler (212)]. Kessler has also shown that DNP blocks NO_3 reduction in *Chlorella* and *Ankistrodesmus* grown aerobically in the dark but not when grown anaerobically. Nitrate reduction by *Ankistrodesmus braunii* is sensitive to pH values below four. In the dark there is an accumulation of NO_2 which is high enough in concentration to be lethal at pH 3.5 (207, 209). In algae containing hydrogenase, NO_2 and hydroxylamine were both reduced directly (213). Kessler has shown wide variations among species of green algae in their NO_3-reducing capacities and has characterized their responses under a number of environmental conditions and in the presence of inhibitors (208). That the content of cells may also have subtle effects on reproductive processes has been shown by Sager & Granick (352) who observed maximum sexual activity in *Chalmydomonas* when the cells had a minimal supply of N in the medium. The selection of a suitable strain for studies of N metabolism should be determined by the problem being examined.

The amino acids of the algae have been determined to be representative [Schieler *et al.* (354); Coulson (66); Ericson & Carlson (92); Magee & Burris (261)]. Fowden (116) has found a more complete list of essential amino acids in *Chlorella* than had been reported earlier by Eny (87). Schieler *et al.* (354) isolated 18 ^{14}C-labeled amino acids from *Chlorella,* but the carbon atoms from $^{14}CO_2$ and labeled glucose were transformed into amino acids by entirely different pathways. Working with cell-free enzymes from *C. vulgaris,* Millbank (267) found aspartic and glutamic acids and alanine most active in transamination. Glutamic acid decarboxylase has also been shown to be active in *Chlorella* [Warburg *et al.* (428)]. Myers & Walker (280) and Walker (423) have identified arginosuccinic acid synthesized from arginine and fumarate in *Chlorella.* In a similar reaction canavanosuccinic acid has also been found [Walker (426)]. Both canavanine and homoarginine are antimetabolites of arginine and lysine in yeast and algae (426). The guanine antagonist, 8-azoguanine, blocks nucleic acid synthesis in *Stichococcus subtilis* [Arnow *et al.* (24)]. The

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nature of the reaction is unknown, and there is no proven use of exogenous purines in the algae.

The N metabolism of *Scenedesmus,* a representative green alga, is strongly oriented toward protein synthesis even under severe nutrient deficiencies. Consequently only small amounts of free, soluble N compounds are normally found in the cells [Thomas & Krauss (413)]. The proteins of *Chlorella, Anabaena, Navicula,* and *Tribonema* have been hydrolyzed for quantitative amino acid analysis. There was a close similarity between the proteins of the algae and such different structures as legume leaves, potato tubers, and *Nitrosomonas* [Fowden (118)]. A similar complement of amino acids was found in the large marine algae [Coulson (67)]. The age of *Chlorella* had little effect on the protein composition except for a significant increase in the histidine content (117). Ten of the essential amino acids constituted 50 per cent of the total amino acids in the algae. The analyses of the chromoproteins of phycocyanin [Wassink & Ragetli (432)] and phycoerythrin [Sisakjan (370)] have been compared by Fujiwara (130) who found that in both the amide content was high; alanine was the greatest in molar concentration and arginine was the greatest in weight. This level of basic arginine may account for the isoelectric point of chlorophyll-containing bodies of *Oscillatoria* being more alkaline than the protein of the surrounding cytoplasm [Yu & Wang (466)]. The relationship between protein and RNA in *Polytomella caeca* has been shown to change with changes in growth rate. Protein synthesis for a given quantity of RNA is more efficient at high rates of growth [Jeener (190, 191); cf. also Iwamura (185)].

Carbon Assimilation and Respiration

Studies of algal respiration have generally been conducted by means of manometry through feeding of exogenous compounds, by analyses of intermediates, and infrequently with the use of cell-free enzyme preparations. The operation of glycolysis and the Krebs Cycle have been well established in the algae. There have been identified all of the intermediates of the direct oxidation of glucose through the pentose phosphate shunt, as postulated for photosynthesis by Calvin and co-workers (30). Whether the gluconic acid shunt is common to both respiration and photosynthesis is not yet established. Details of the assimilation of organic C in most cases have been assumed to be similar to the system identified in other organisms, but complete demonstration of many of the requisite reactions have not been clearly demonstrated in the algae. This is especially true of algae which are able to assimilate such compounds as sterols, fatty acids, alcohols, and aldehydes.

The interrelationships between photosynthesis and respiration are elusive, but some approximation of the mode of coexistence can come from sifting the evidence dealing with both processes. Benson & Calvin (35) have shown, by the absence of ^{14}C in Krebs Cycle intermediates in illumi-

nated algae supplied $^{14}CO_2$, that PGA is not channeled into this respiratory cycle. It seems clear that in most cases respiration is as rapid [Brown (47)] or even more rapid [Brown & Webster (49)] in the light as in the dark. There is some evidence that respiration taking place primarily in mitochondria outside the chloroplast draws heavily on the polysaccharide reserve or possibly even on glutamic acid through protein hydrolysis as suggested by Steward & Thompson (385). Little or no ATP formed directly in photosynthesis can flow to the energy-consuming systems directly dependent on respiratory phosphorylation since oxidative metabolism would probably be inhibited in light. Only in the dark is PGA liberated from the photosynthetic cycle of the chloroplast to the respiratory centers. The two processes (whether both in the chloroplast or not) can be considered independent although they are probably linked indirectly [cf. Good (151)]. In the light, photosynthesis is the dominant reaction being as much as 20 times as active as respiration which may draw on the reserve of starch or sugar even at some loss in efficiency. Only one-eighth to one-ninth of exogenous glucose is used in respiration [Syrett (392)], so starch and protein syntheses proceed at the expense of respiration or proceed as the primary reaction sequence after phosphorylated hexose has saturated respiration. When only endogenous respiration remains, after the exhaustion of exogenous sources, the primary reaction sequence is respiration.

Of the C sources for algae, CO_2 is probably the commonest, but there remains considerable disagreement as to the form in which it penetrates the cell or chloroplast. Algae growing at alkaline pH levels are exposed to limited amounts of gaseous CO_2 but to large amounts of bicarbonate. Earlier data indicated that *Scenedesmus* absorbs both CO_2 and HCO_3^- but that *Chlorella* required carbon dioxide [Osterlind (293)]. Further experiments confirmed the capacity of *Scenedesmus* to utilize HCO_3^- but only in "young" cultures (294). Furthermore, proper photoactivators of a factor necessary for HCO_3^- assimilation are necessary (295). The inhibition of photosynthesis observed when algae are changed from high CO_2 concentration to low may also be due to the inhibition of HCO_3^- absorption (298). Showing that high CO_2 levels serve as growth inhibitors through toxic effects on photosynthesis, Nielsen (285) pointed out that the greater the diffusing distance between the cell surface and the cell membrane the greater the requisite concentration of carbon dioxide. However, in some cases a concentration as low as one per cent was inhibiting. The effects of pH should not be overlooked in such studies (284). Carbon dioxide has also been shown to stimulate dephosphorylation in *C. pyrenoidosa* subsisting on endogenous respiration [Schlegel (355)]. In contrast, Myers (275) has reported that in the light, glucose inhibits CO_2 assimilation although the growth rate of *Chlorella* on glucose and CO_2 in the light is as great as on CO_2 alone. In mixotrophic growth half of the cell carbon was from CO_2 and half from glucose. Carbon dioxide fixation is not restricted to the light. Ulubekova & Kusmina (418)

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found that dark reduction of CO_2 in H-adapted algae was two and one-half times as fast as photoreduction when hydroxylamine blocked further photosynthesis. Lynch & Calvin (259) demonstrated that CO_2 fixation coupled to energy obtained from respiration in the dark is greater in *Euglena* than in any other plant or animal. This dark fixation yields the same intermediates as photosynthesis. Brief evolution of CO_2 in the light and absorption of CO_2 in the dark has been established by Brown & Whittingham (50) for *Chlorella* during alternating periods of light and darkness.

A wide variety of organic C compounds are assimilated by algae or appear as intermediate products at some stage of growth. Tabular data listing organic C sources for algae are given by Albritton (3). However, more often than suspected in many studies, media containing sugars and other organic compounds may have been inactivated or may have reacted to form toxic compounds during autoclaving [Patton & Hill (301)]. Acceptance or rejection of carbon sources from experiments with mixed media must be done with caution. There is no common pattern in the assimilation of C compounds. Algae range from the versatile which assimilate many, to the fastidious which assimilate few C compounds, and to the ultimate obligate autotrophs [Lewin (241)] which use only carbon dioxide. The obligate autotroph apparently depends on photosynthesis in some way to alter the physicochemical nature of the cell thus making growth possible (441). Such a dependence may be partially reflected in some strains of *C. vulgaris* [Finkle *et al.* (97); Killam & Myers (214)] in which only catalytic amounts of light are required for efficient heterotrophic growth. A critical action spectrum for this effect is badly needed. Even in *C. pyrenoidosa* growth is better if some light is available even though the medium is adequate for heterotrophic growth [Kandler (199)]. The dependence on light for substrate utilization has often been overlooked in studies of heterotrophy because of the desire to eliminate all photosynthetic contributions to growth.

Reactions other than those associated with C assimilation may be light-sensitive. In experiments with *Oedogonium,* Bühnemann (52, 53, 54) observed a rhythmic diurnal sporulation which he attributed to alternating synthesis and destruction of an inhibitor. The effects of light are complicated, but blue wavelengths appear to be especially potent for intensifying the cyclic process. The daily photosynthetic rhythm of *Hydrodictyon* is controlled by both blue and red light [Pirson & Schön (307)]. Inhibiting effects of light are also known. With blue light, Kandler & Schotz (203) have been able to block the assimilation of glucose by alpha- and beta-carotene-deficient strains of *Chlorella.* The well-established responses of *Elodea* protoplasm to small amounts of light probably have their counterparts in the algae. Light intensity and quality may be governing factors in the metabolism and their interaction cannot be overlooked in interpreting the results of experiments on C nutrition [Kok (221, 222)].

Extensive listings of organic compounds serving as C or energy sources

for algae include most of the common sugars, alcohols, and Krebs Cycle acids [Neish (282); Kandler & Ernst (202); Eny (88, 89, 90); Schlegel (356); Lewin (242)]. Ordinarily, undissociated molecules are considered to penetrate membranes readily, but Erickson *et al.* (91) found that stimulation of O_2 uptake in *Chlorella* is related to the concentration of the acetate anion, although inhibition of respiration at higher concentrations was related to the number of undissociated molecules. In demonstrating the respiration of Krebs Cycle acids, as well as butanol, ethanol, and propionate by *Euglena,* Danforth (69) also observed that pH made considerable difference on the permeability of the membrane. Increased assimilation was not always associated with higher pH. Schöu *et al.* (358) identified all of the photosynthetic intermediates synthesized from exogenous glycolic acid and Milhard *et al.* (266) found lipides and all of the Krebs Cycle acids when either pyruvic or hydroxypyruvic acids were assimilated. Exogenous pyruvate was transformed to Krebs Cycle intermediates as rapidly in the light as in the dark, but more pyruvic acid and alpha-ketoglutaric acid were found in *Chlorella* growing heterotrophically on glucose than when growing phototrophically [Millbank (268)]. Grosskinsky *et al.* (156) were able to demonstrate growth of *Chlorella* on 1,3-butandiol and glycerol. Inhibition of substrate utilization by poisons may be influenced by light. Kandler (200) has shown that of a series of inhibitors only arsenate and *o*-phenanthroline inhibit glucose utilization and synthesis in both light and dark, and Holzer (177) found inhibition of *C. pyrenoidosa* with concentrations of 2,4-DNP in the light which had no effect in the dark. The amount of aeration also affects substrate utilization, but Taylor (409) showed that this is primarily a matter of agitation, for increases in the O_2 tension made no difference in glucose utilization.

Peculiarities in nutrition are frequent among the various algal classes. Of the chrysomonads, *Ochromonas malhamensis* is capable of utilizing both CO_2 and glucose in the light but growth is only marginal during strictly autotrophic nutrition, thus indicating only a minimum contribution of photosynthesis [Myers & Graham (277)]. Reazin (334) has shown that light maintains cofactors necessary for carbohydrate assimilation. A lag in subsequent glucose assimilation results after cells are starved in the dark. Here, the pathway for glucose utilization appears to be that of Myerhof and Embden rather than the pentose pathway that might seem to be indicated by the light requirement [Reazin (335)]. Substrates for *Ochromonas* are not restricted to glucose. Pringsheim (318) has obtained growth of nine strains in the dark on glucose, fructose, mannose, galactose, sucrose, maltose, raffinose, starch, glycogen, glycerol, and ribose, but not on lactose, cellobiose, inulin, mannitol, pyruvic acid, glyceraldehyde, arabinose, xylose, and yeast homogenate. Hutner *et al.* (181) found that the minimum requirements for thiamin, cyanocobalamin, biotin, and a carbohydrate or glycerol were the same in light or darkness for *Ochromonas* and *Poteriochromonas*. The

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nutrition of *Polytoma, Polytomella, Chilomonas,* and *Euglena* gives evidence of considerable versatility by inclusion not only of sugars and Krebs Cycle intermediates but also of aldehydes, acetones, long-chain acids, and alcohols [Reinhardt (338); Pringsheim (319); Holz (176); Danforth & Wilson (70); Cirillo (58); Wise (451)]. Provasoli & McLaughlin (326) reported a marine flagellate in which a fat requirement was filled by cholesterol, cholesteryl laurate, cholesteryl glycoside, 5-dihydroergosterol, esterone methyltestosterone, zymosterol, taurocholic and glycocholic acids, and oleic acid derivatives. A steroid requirement in *Peranema* was satisfied by cholesterol plus lecithin [Storm & Hutner (387)]. Allen (14) has studied C sources for a number of species of blue-green algae, and Kratz & Myers (224) have established *Anabaena variabilis, Anacystis nidulans,* and *Nostoc muscorum* as obligate autotrophs. On the other hand, Allison *et al.* (19) show some use of acetate by *Nostoc* which is accelerated by concurrent photosynthesis.

Endogenous respiration appears to be a system quite different from that respiration achieved when adequate photosynthate, or exogenous C, is present. Pirson *et al.* (306) found endogenous respiration to be stimulated by glucose, but the substrate oxidized was reserve material from previous photosynthesis and not exogenous glucose. Endogenous respiration is not sensitive to HCN but glucose respiration in *Scenedesmus* and *Chlorella* and anion absorption are blocked by 3 to 10 μM [Osterlind (296, 297)]. Syrett (392), also finding the same pattern of HCN inhibition, suggested that assimilation and oxidation of glucose are coupled by a cyanide-sensitive respiratory system. Recently Geoghegan (139) confirmed this observation with *Chlorella viridis* and found also that CMU and DNP inhibited the same system. The site of action of HCN is normally expected to be cytochrome oxidase, but the evidence above and the observations of Webster & Frenkel (438) that respiration in *Anabaena* is inhibited by HCN, azide, and *o*-phenanthroline but not by CO, make the specificity of action questionable. Either HCN is less specific or independent terminal oxidase systems exist in algae.

The preparation of cell-free extracts for enzymatic studies of respiration has proved difficult. Millbank (269) reviewed the methods available for extraction from *Chlorella,* but reported that only one procedure, employing partially dried cells, was satisfactory. He was able to show oxidation of Krebs Cycle acids with such preparations. Richter (344) attempted the separation of catalase and other enzymes from *Hydrodictyon* by paper chromatography but the results were not good. Holzer & Holzer (178) have been successful in demonstrating degradation of carbohydrates via phosphorylated intermediates with ATP and acetone extracts of *Chlorella.* The nucleotide coenzymes, UDP-glucose and UDP-galactose, have been identified in *Scenedesmus* [Buchanan *et al.* (51)], and glucose-6-phosphate dehydrogenase, aldolase, triose phosphate dehydrogenase, and malic dehy-

drogenase have been demonstrated in *Ulva* [Jacobi (187)]. Isocitric dehydrogenase in *Ulva* and *Ceramium* and 6-phosphogluconate dehydrogenase in *Tolypothrix, Ceramium,* and *Ulva* have also been established [Cohen (62); Mehler (264)]. Evidence for direct oxidation of glucose and galactose to aldonic acids, well documented for *Iridophycus* by Bean and co-workers (32, 33, 34), should be sought in the fresh water algae.

The effects of ultraviolet radiation on respiration support the hypothesis of independent and different endogenous and exogenous respiratory systems. Holt & Arnold (174) found glucose respiration four times more susceptible than endogenous respiration in *Chlorella.* This was confirmed with *Scenedesmus* (175) and in a more detailed study with *Chlorella* [Redford & Myers (336)]. Gessner & Diehl (145) and Biebl (37) correlated the ultraviolet resistance of different algae with the depths at which they normally grow. Deep-water forms were much more susceptible than *Chlorella,* an alga representative of those found near the surface.

Vitamins, Hormones, and Antibiotics

Culture of many fastidious algae is possible only with media supplemented with vitamins. Information concerning the vitamin titre of natural waters may, therefore, be as useful in predicting algal populations as nutrient assays [Hutner *et al.* (182)]. Thiamin, cobalamin, and biotin are most commonly required by auxanotrophs. The flagellates have been implicated most frequently [Droop (83)], and Provasoli & Pintner (329) tabulate the requirements of 24 species in four algal divisions. Rigorous techniques are dictated for removing contaminant vitamins from agar [Robbins (345)], media, and culture tubes; consequently requirements are often satisfied accidentally. Of the vitamins, B_{12} has received the most attention in the algae. Clearcut establishment of a B_{12} requirement is complicated by the fact that a number of active, pseudovitamin B_{12} factors will satisfy the cyanocobalamin need in many algae. *Euglena gracilis* var. *bacillaris,* used as an assay organism [Witkus *et al.* (452); Robbins *et al.* (346, 347)], is now known not to be specific for B_{12}. An absolute requirement does exist in *Ochromonas, Poteriochromonas,* and *Synura* [Hendlin (168); Barber *et al.* (28)], *Monochrysis* [Droop (82)], and *Skeletonema* [Droop (81)]. Further complications come from the evidence of Ford *et al.* (109) that pseudovitamin B_{12} competitively inhibits the use of B_{12} by *Ochromonas,* and that filtrates of *Ochromonas* and *Euglena* cultures sequester B_{12} and make it unavailable. The B_{12} requirement in dependent species also varies. Lewin (250) found the requirement for *Stichococcus cylindrica* to be 0.2 μg./l. or 50 molecules of cyanocobalamin per cell, in contrast to 4900 required by *Euglena. Gymnodinium* is satisfied by only 0.001 to 1.0 μg./l. [Sweeney (391)]. Though its role is still unknown, the occurrence of the requirement in species of five algal divisions [Ford & Hutner (110)] suggests a central role in metabolism. The 12 per cent decrease in nucleic acid

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found in B_{12}-deficient *Euglena* by Soldo (373) hints at some relation to nucleic acid synthesis. Although B_{12} is missing from higher green plants, *Chlorella* contains 6.3 μg. cobalamin/100 gm. dry weight [Brown *et al.* (48)] and the blue-green alga, *Anabaena,* yields ten times this amount of which 65 to 70 per cent is cobalamin and the remainder is other active factors. Reviewing the literature, Darken (72) listed *Calothrix parietina* as the richest algal source with 0.64 μg. B_{12}/gm. dry weight.

Less effort has been devoted to the other vitamin sources or requirements. Combs (64) has published analyses showing high levels of ten vitamins in *Chlorella.* The operation of PAB in the cell has been inferred from the inhibiting actions of sulfanilamide on *Nitzschia* [Wielding (445)], *Chlamydomonas* [Lewin (248)], and *Scenedesmus* [Krauss & Thomas (233)], although a requirement has not been found for any species. Growth of *Scenedesmus* has been reported to be stimulated by ascorbic acid [Posey (311)]. Oxidation of ascorbic acid has been blocked *in vitro* by sodium alginate [Ericson & Gasparetto (93)]. Thiamin, commonly required by unicells, is synthesized by young thalli of marine algae and by *Scenedesmus* [Gerdes (142)]. Ten strains of *Scenedesmus* contained between 0.3 and 10.5 μg./gm. dry weight. The vitamin, synthesized only in young cultures, was produced in the dark and destroyed in the light regardless of the length of the light and dark periods. Vitamin K activity corresponding to 6 μg. menadione per gm. dry weight in *C. vulgaris* has been reported by Dam (68). Pantothenic acid can be a N source for *Scenedesmus* [Algeus (11)] but probably has no role as a vitamin. Pyridoxine appears to act as a coenzyme in the transamination of aspartic acid [Algeus (9)].

It is logical to suspect that algae produce hormones or growth substances which govern various phases of vegetative and reproductive life, and which may influence other microorganisms. The most detailed papers on this subject are those published by Moewus to the effect that sex hormones regulate the attraction and fusion of gametes. These studies are ably reviewed by Raper (333) and Smith (371, 372). Unfortunately for the very intriguing biochemical sequences of the Moewus scheme, key experiments have not been satisfactorily repeated and the work has been subject to severe attack [Lewin (252); Ryan (349); Förster & Wiese (112)]. Recognizing serious faults in the proposed system, Hartmann (161, 162) has defended the concepts of bipolar bisexuality, bisexual potency, relative sexuality, and the existence of gamones in cell-free filtrates. Förster and co-workers (113, 114) have found an agglutination gamone, which is identified as a glycoprotein, in the tips of the flagella in *C. reinhardti,* but it bears no relation to the carotenoid system of Moewus. A copulation factor in the diatom *Cocconeis* [Geitler (137)] and a "swarming substance" which regulates colony formation in *Pediastrum* [Moner (270)] have also been found. Repeated studies of the effects of filtrates on gametes should remove many of the present misconceptions.

The action of auxins and related substances, first identified in higher plants, has not been exhaustively studied in the algae. Brannon (44) and Brannon & Bartsch (45) investigated their effects on *Chlorella, Coccomyxa,* and *Mesotaenium.* They obtained stimulation of growth with NAA, IAA, IBA, and PAA in inorganic but not in dextrose media. It is quite possible that there was a contribution to C nutrition rather than hormone action per se. Growth stimulation, principally by IAA, has been reported for *Scenedesmus* [Yamafuji & Nakamura (463)], *Chlorella* [Yin (464)], *Codium* [Williams (447)], *Acetabularia* [Dao (71)], *Phycopeltis* [King (215)], and *Rhizoclonium* [Davidson (73)]. An inhibitory action of 2,4-D on photosynthesis of *Chlorella* has also been reported [Wedding *et al.* (439); Erickson *et al.* (91)]. It is not safe to conclude that the observed effects are the results of the same regulating function established in higher plants rather than quite unrelated phenomena.

Since the identification of the autoinhibitor, chlorellin, in filtrates of *Chlorella,* many reports of the secretion of antibiotics have been published. Harder & Oppermann (160) obtained a yellow secretion of *Stichococcus* and Protosiphon which is bacteriostatic for Gram-positive and Gram-negative bacteria. Nielsen (283) maintained that antibiotics produced by *Chlorella* and *Thalassiosira* suppress bacterial respiration. McVeigh & Brown (263) and Proctor (323) have studied the interaction of *Chlamydomonas* and *Haematococcus* when cultured together. Stimulating effects of bacterial dialysates have also been shown for both organisms (263). Activity of filtrates of many species of algae against other species is frequent [Rice (342), Lefevre & Nesbit (239); Jørgensen (196); Jakob (188, 189); Lefevre *et al.* (238)] and Mucibabic (272) has found interaction between *Tetrahymena* and *Chilomonas.* Ruschmann (348) has followed the shift in the contaminating bacterial flora as cultures of algae age, presumably because of disappearance of specific antibiotics. In no case have the chemical compounds been identified. Furthermore, no intracellular growth modifiers have ever been clearly established as operating in natural populations [Talling (401)].

Commercial antibiotics, often employed in purifying algal cultures, have been studied from other points of view. Permanent chlorophyll-less races of *Euglena* [Provasoli *et al.* (324, 325)] and *Chlorella* [Dube (84)] have been created by treatment with streptomycin. Pringsheim & Pringsheim (321) have also been able to achieve colorless mutants readily by incubation of *Euglena* at slightly suboptimal temperatures without streptomycin. The active, energy-requiring absorption of streptomycin in *Nitella* has been found to have an optimum at pH 5 and an activation energy of 7500 cal./mole mediated by an ion-binding carrier system [Litwack & Pramer (256); Pramer (312, 313)]. Considerable differences exist in the concentrations of antibiotics which inhibit different species [Barkley (29)]. The substrate on which the alga grows will have a major effect on the potency of toxic

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agents [Galloway (134)]. Screening studies, including most of the available antibiotics, have been done on algae [Zehnder (467); Tomisek *et al.* (416); Galloway (134); Foter *et al.* (115)], but little is known about the mechanism of action. Examination of the role of galactose in protecting against Polymyxin B toxicity in *Scenedesmus* has indicated that one site of inhibition is glucosephosphate isomerase [Krauss & Galloway (231)].

Pigments

The green, yellow, blue, and red algal pigments have attracted the attention of organic chemists and their composition is comparatively well known [Strain (388)]. The roles of all the chloroplast pigments are associated with photosynthesis, chlorophyll-*a* being activated by energy absorbed by the other chlorophylls, carotenoids, phycocyanins, and phycoerythrins. Details of the energy transfer have been studied in marine algae [Blinks (39)] and diatoms [Tanada (408)]. The energy-absorbing role is probably not exclusive but no other function has been established. The abundance of carotenoids outside of the chloroplasts in *Trentepholia* and in localized parts of blue-green algae [Geitler (138)] does suggest secondary roles. The orientation of the pigments within chloroplasts, especially of *Euglena* and *Poteriochromonas,* has been the subject of papers by Wolken and coworkers (455 to 461). Their picture of the chloroplast of *Euglena,* which probably should be called a single granum [Thomas *et al.* 412)], is that of a laminated structure composed of a 250 Å layer of protein and lipoids bordered by monomolecular 60 Å layers of chlorophyll. Each such unit is separated from adjoining ones by a 200 to 250 Å layer of aqueous protein. This laminated structure, visible under the electron microscope, collapses in the dark. The action spectra for both chlorophyll and carotenoid resynthesis after dark periods have proved to be identical (457). The importance of phycocyanin orientation in the grana of *Synechococcus* has also been established by Thomas and De Rover (411), who observed failure of the capacity of the grana to support the Hill reaction after loss of part of the phycocyanin. Techniques for the extraction of the intact chlorophyll-protein complex from blue-green algae [Silberger & Haxo (366)] and from *Chlamydomonas* [Sherratt & Evans (362)] make possible *in vitro* studies of the chromoprotein.

Light, temperature, and nitrogen levels modify the genesis of pigments. Aach (2) reports that the yellowing of cultures in bright light is due to a more rapid rate of chlorophyll destruction than of carotenoid oxidation. Bright light does bring about carotenoid destruction as cultures age [Levy (240); Goodwin (152)], but Kingsbury (216) has used the carotenoid content of blue-green algae as a satisfactory measure of growth. Droop (80) found that carotenogenesis is independent of available N, but not of the C supply, and that cell multiplication in *Haematococcus* precludes carotenoid accumulation and will cause breakdown of presynthesized reserves. Claes

(60) has shown a dependence of *Chlorella* mutants on light for the complete synthesis of carotene precursors produced in the dark. Isomerization of poly-*cis*-carotenes has been specifically attributed to blue light (59). Phycocyanin and chlorophyll ratios vary with light and temperature in blue-green and red algae. Myers & Kratz (278) found a maximum of 2.8 per cent chlorophyll-*a*, 1.2 per cent carotene, and 24 per cent phycocyanin in cells of *Anacystis* grown at low light intensities and high temperature. Similarly in *Porphoridium cruentum*, phycoerythrin and chlorophyll were both shown to be higher in weak light although phycoerythrin was proportionally higher than chlorophyll [Koch (220)].

Products of Metabolism

Among the carbohydrates and their derivatives in the algae, special attention has been given the polysaccharides by Frederick. A cell-free extract from *Oscillatoria princeps* synthesized from glucose-1-phosphate a glycogen-like polysaccharide containing 14 to 16 glucose units (119). Low temperature changed the type of polysaccharide synthesized by variants, or unstable mutants, which synthesized amylopectin-like polymers with few alpha-1:6 linkages (120, 121, 122, 125). The Michaelis-Menton K_s of the phosphorylase is less than that for the branching enzyme (123) which was found in higher concentrations in the normal strains (124). Frederick concludes that the differences in polysaccharides of *Oscillatoria* depend primarily on the concentrations of the branching enzyme (126). Hough *et al.* (180) also found amylopectin-like polymers in *Oscillatoria*, but *Nostoc* yielded a mucilaginous, complex, acidic polysaccharide composed of at least six different monosaccharides. The polysaccharide reserves of the green algae, as high as 20 per cent of the dry weight in *C. vulgaris* (26), are composed of amylose and amylopectin. Glattfeld (146) has crystallized alpha *d*-glucose from the hydrolysate of *Scenedesmus*. Bailey & Neish (26) contradicted the earlier reports of cellulose and chitin in *Chlorella* [Wurdack (462)] by finding none of either. They were able to obtain both phosphorylase and a branching enzyme from cell-free extracts. Kreger & Meeuse (234) employed x-ray powder diagrams to identify paramylon as a beta-1:3 glucose polymer identical to yeast hydroglucan. Singh (368) identified the paramylon of *Trachelomonas* as a similar glucan. Pringsheim (320) has reported that paramylon accumulates only in the absence of growth, and can be found outside the chromatophores in *Euglena* as is starch in *Cryptomonas* (316). Leucosin, the Chrysophycean reserve, is also a short-chain glucose-polymer consisting of only 8 units but otherwise similar to laminarin (420) [see also Quillet (332)]. In diatoms, Barachkov (27) identified galactose, glucose, xylose, rhamnose, mannose, and arabinose in hydrolysates although cellulose and hemicelluloses were absent. In his analysis of the flagella of *Polytomella*, Tibbs (414) has indicated a carbohydrate level of 6.2 per cent. The preparation of flagella completely free of all other cell structures was

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probably not possible. In green algae no component, except possibly sucrose, has been found which is at all analogous to α-D-galactosyl-2-glycerol phosphate found in *Iridophycus* (31).

Lipide accumulation of algae may be accelerated by N deficiency, but the red and blue-green algae contain high fat levels even in N-sufficient media. Within the cell there appears to be no correlation between unsaponifiable lipoid and either fatty acid or total nitrogen [Collyer & Fogg (63)]. Aach (1) has charted shifts in lipoid levels in green algae finding 43.4 per cent of the dry weight of N-starved *Scenedesmus* to be lipide. Of these 2.1 per cent were phosphatides—a group of compounds studied very little in the algae. Kathen's (204) analyses of the fats of *Chlorella, Scenedesmus,* and *Nitzschia* indicated that they were all of a composition similar to that of seeds and heterotrophic microorganisms. Analyses of the unsaturated fatty acids of *Chlorella* have been published by Paschke & Wheeler (299). Fogg (105) has related fat formation to photosynthesis by following ^{14}C assimilation in diatoms, but fat synthesis also proceeds in the absence of photosynthesis [Coulon (65)]. Sterols have been less frequently studied than fats. A sterol similar to chondrilasterol was reported from *Scenedesmus* (232), and low sterol levels (0.009 to 0.6 per cent) were found in diatoms [Low (258)].

Early reports [Krogh *et al.* (235); Aleev (4)] of secretion of organic materials have been confirmed in recent studies of many genera. Goryunova reported excretion of oxalic, citric, tartaric, and succinic (153), acetic, formic, and presumable pelargonic acids (154), and acetaldehyde by *Oscillatoria.* The reports of N secretion by blue-green algae have been supported by Fogg who found polypeptide and amide secretion by young cultures of *Anabaena* (103), and liberation of N compounds to adjacent cells during heterocyst formation (101). Forrest *et al.* (111) have also observed a rapid loss of pteridine from a blue-green alga when the temperature was dropped to 4°C. Among the green algae, Tolbert & Zill (415) reported that 3 to 10 per cent of the total C fixed by *Nitella* was secreted into the medium as glycolate. Polysaccharide and organic acid secretion are also well established for *Chlamydomonas* [Lewin (253); Allen (15)]. *Synedra* excreted lipoids equivalent to 10 per cent of the dry weight of the cells during culture [Goryunova (155)]. Slight peptide, amide, and amino-N secretion has also been shown in *Chlorella* and *Chlamydomonas* by Fogg & Westlake (108) who emphasized the importance of such secretions in the economy of fresh waters.

Of the topics relating to algal physiology, but beyond this review, those dealing with physiological genetics should be mentioned. They give promise of significant contributions to the understanding of metabolism. Mutants, or genetically modified strains, of the heterothallic, sexual genus, *Chlamydomonas,* have been obtained by chemical treatment [Sager (350); Wetherell & Krauss (442)], by ultraviolet irradiation [Lewin (249, 251);

Eversole (94); Ebersold (85)], and by x-radiation [Nybom (289); Wetherell & Krauss (443)]. A basis for the further study of inheritance in such organisms has been established. Other mutants of nonsexual genera [Herbst (170); Hammerling (159); Davis (74); Schoenborn (357); Demeter (79); Bogorad (40)] have proved useful in determining steps in synthesis or degradation of organic components of the algae.

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THE METABOLISM OF AMINO ACIDS AND PROTEINS IN PLANTS[1,2]

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In a recent review Steward & Pollard (149) have broadly surveyed the progress which has been made during the past ten years in our knowledge of the metabolism of nitrogen in plants. They emphasize particularly the rapid advances which have resulted from the development of chromatographic methods of analysis and from the use of isotopically labelled nutrients as a means of exploring the pathways of cellular metabolism. Nevertheless, many of the basic problems of the metabolism of amino acids, and especially their relation to the synthesis of proteins, remain uncertain and obscure. There is in fact no general agreement on whether amino acids are precursors in the synthesis of proteins in plants; different approaches to this problem are clearly evident in the reviews of Street (153), Wood (183), Webster (175) and Steward & Pollard (149).

From evidence such as that outlined by Wood (183) and by Webster (175), it has been generally assumed that amino acids participate directly in the synthesis of proteins although much of this evidence is circumstantial rather than decisive. For example, the requirement of amino acids as specific nutrients for biochemical mutants of fungi and other heterotrophic microorganisms is well established and is fully consistent with the view that amino acids are essential intermediates in the synthesis of proteins. Similarly C^{14}-labelled amino acids have been shown to be incorporated into proteins both in cell-free preparations from higher plants and in intact plant tissues although critical evidence of the relation between incorporation and the normal processes of protein synthesis is lacking.

An alternative hypothesis in which amino acids are not regarded as precursors in the synthesis of proteins in plant cells has been developed and elaborated by Steward and his collaborators [see for example Steward & Street (151) and Steward & Pollard (149)]. It is suggested that free amino acids arise mainly from the catabolism of proteins whereas protein synthesis results from *en bloc* condensations of reactive intermediates, derived from carbohydrate, with nitrogen donors such as glutamine and glutamic acid. The underlying biochemical mechanisms have not been precisely

[1] The survey of literature pertaining to this review was completed in October, 1957.

[2] The following abbreviations will be used: ADP, adenosinediphosphate; AMP, adenosinemonophosphate; ATP, adenosinetriphosphate; DNA, deoxyribonucleic acid; DPN, diphosphopyridine nucleotide; HDP, fructose-1,6-diphosphate; RNA, ribonucleic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid.

[3] The authors wish to thank their colleague, Dr. A. J. Willis, for his critical reading of the manuscript.

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formulated; little support has been found for the view of Linderstrøm-Lang (88) that ketopeptides, formed by condensation of keto aldehydes and keto acids with amino acids, are intermediates in protein synthesis.

In this review an attempt will be made to evaluate the recent evidence upon the synthesis and interconversion of amino acids in plants, and, in particular, upon the relation of amino acids to the metabolism of proteins; the relative merits of the "amino acid" and "alternative" hypotheses of protein synthesis will be examined.

One of the important lines of evidence which has been put forward in support of the alternative hypothesis is the close relation between protein metabolism and respiratory activities in plant tissues. The concept of a continuous protein cycle in which most of the carbon dioxide of cell respiration is derived from the catabolism of amino acids can be traced historically from the writings of Borodin (17) to its modern development and elaboration by Gregory & Sen (59) and by Steward and his associates (149, 151). Besides its implications with regard to protein metabolism and cell respiration, the concept of a continuous protein cycle has an important bearing on the mechanisms and regulation of carbohydrate breakdown. It is appropriate, therefore, in this review to consider recent evidence concerning the interrelations between respiratory processes and the metabolism of carbohydrates and proteins in plants.

Assimilation of Nitrogen and the Primary Synthesis of Amino Acids

Since the publication of the comprehensive reports edited by McElroy & Glass (95) in *A Symposium on Inorganic Nitrogen Metabolism*, further progress has been made in several directions in the study of the initial stages in the assimilation of nitrogen. The enzymic mechanisms and some of the intermediate steps in the reduction of nitrate have been examined. Further light has been thrown on the first products of assimilation during the utilization of ammonium salts and nitrates by higher plants and during the fixation of elementary nitrogen.

Fixation of nitrogen.—A detailed study of the fixation of nitrogen by *Azotobacter vinelandii* with N^{15} as a tracer has been described by Allison & Burris (3). They followed the incorporation of the isotope into ammonia and into amino acids which were separated by chromatography on ion-exchange resin. The cells were rapidly killed by acidification after short periods of exposure to N^{15} and the supernatant and cell residues analysed. Some difficulties in the interpretation of the results arose from the random mixing of the nitrogen of the medium with that of the soluble cell constituents. However, the data yielded unequivocal evidence that amide nitrogen and ammonia increased most rapidly in N^{15} abundance and showed other features typical of primary products of fixation. Of the free and combined amino acids, glutamic acid was enriched by N^{15} most rapidly; it had an appreciably higher abundance than aspartic acid and neutral and basic amino acids. The experiments gave strong evidence that ammonia

was the chief, if not the only, primary product of fixation in the cells. From the rapid excretion of fixed N^{15} into the medium in their experiments, Allison & Burris suggested that the reduction processes of fixation occur on the cell surface, and they discussed the possibility that special structural requirements may be needed for the coupling of dehydrogenase activity with other oxidation-reduction processes.

Investigations of cell-free preparations of *Azotobacter* by Burma & Burris (22) have thrown light on other aspects of nitrogen assimilation by this organism. With preparations made by sonic disintegration, they have shown that the incorporation of $N^{15}H_3$ was greatly increased in the presence of α-ketoglutarate and that glutamic acid was most highly labelled with the isotope. High rates of transamination between N^{15}-glutamic acid and other acids were observed. When N^{15}-glutamic acid and a complement of other amino acids, ATP, and HDP were added to the cell-free preparations, heavy labelling of protein occurred; the labelling was almost entirely confined to the glutamic acid in the protein. The evidence now available strongly suggests that in *Azotobacter* the chief pathway for nitrogen fixation involves reduction to ammonia which is then rapidly utilized in the synthesis of glutamic acid.

The mechanism of the reduction of gaseous nitrogen to ammonia is uncertain. The data of Azim & Saraf (7) indicate a rapid reduction of nitrate and nitrite to ammonia in *Azotobacter,* but it is unlikely that the reductases involved play an important part in nitrogen fixation. In experiments with *A. agile* Spencer *et al.* (143) have shown that the adaptive enzymes for nitrate reduction are formed in response to nitrate but not when gaseous nitrogen, ammonium salts or glutamate are supplied. In this connexion it is of interest that the distribution of molybdenum in cell-free preparations of *A. vinelandii* has been shown by Keeler (76) to differ in cells grown on different sources of nitrogen. When cells were grown with gaseous nitrogen, the microsome fraction contained a higher proportion of molybdenum than when cells were grown on nitrate or ammonia. Carnahan & Castle (24) have shown that *Clostridium* has special nutritional requirements associated with the fixation of nitrogen. In a brief report Bach & Burris (8) gave some evidence that in *A. vinelandii* hydrazine may be an intermediate in nitrogen fixation. When hydrazine labelled with N^{15} was supplied to dense suspensions of the organism, a large part of it was recovered as dihydropyridazinone-5-carboxylic acid. The latter compound was found to occur both in *Azotobacter* and in soya bean nodules, suggesting that it is an intermediate in nitrogen fixation.

In other nitrogen-fixing organisms there is now clear evidence that special mechanisms associated with the synthesis of amino acids may be closely coupled with the fixation and assimilation processes. In experiments with *Nostoc muscorum* and other blue-green algae Linko *et al.* (89) have shown that citrulline is a conspicuous product. When the algae assimilated $C^{14}O_2$ for short periods, up to 20 per cent of the radioactivity was contained

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in citrulline and nearly all of this activity was located in the carbamyl group. The synthesis was most active in the light, perhaps because of its dependence on phosphorylations coupled with photosynthesis. It is likely, therefore, that in these organisms as in animal tissues [Ratner (129)] the conversion of ornithine to citrulline by means of carbamyl phosphate or carbamyl glutamate plays an important part in carbon dioxide and nitrogen assimilation. The possibility that the ornithine → citrulline step of the so-called ornithine cycle may be involved in nitrogen fixation in root nodules is also indicated. The presence of relatively large quantities of citrulline in nodules of alder was first shown by Virtanen & Miettinen (173) and it is now well established that nitrogen fixation can occur in these nodules. In fact the extensive work of Bond and his collaborators (14, 15, 16, 57, 93) has provided evidence that nitrogen fixation is widespread in nodules of non-leguminous plants.

Assimilation of nitrate, nitrite and ammonia.—The evidence now available indicates that nitrate can be reduced to ammonia in plants by a series of two-electron steps, coupled with the oxidation of pyridine nucleotides by means of metallo-flavo-protein reductases, with nitrite, hyponitrite, and hydroxylamine as intermediate products. The most thorough study of the enzymes has been made in preparations from *Neurospora crassa*. Nicholas and his collaborators (105 to 108, 117, 118, 119) have described some of the chief properties of the reductases and particularly the dependence of their activities on trace metals. Molybdenum, manganese, and copper, in addition to iron, are involved in one or more steps in the reduction of nitrate in plants. The first separation by Medina & Nicholas (105, 106) of hyponitrite reductase, which catalyses the reduction of hyponitrite to hydroxylamine is of particular interest, since there has been much uncertainty concerning the mechanism of nitrite reduction. Some evidence was obtained that this enzyme from *Neurospora* was a metallo-flavo-protein, and the inhibitory action of dinitrophenol indicated that phosphorylation took part in the reaction.

In many microorganisms some of the reductases involved in nitrate assimilation are adaptive enzymes. The data of Morton (113) concerning preparations from mould fungi indicate that rapid changes may occur in the activity of the nitrate → nitrite reducing system. In these fungi a nitrate reducing system is detectable even when they are cultured in absence of nitrate, but the system undergoes a sharp fall in activity when ammonia is assimilated by the organisms. The nitrate reductase of soya bean nodules, studied by Cheniae & Evans (27), does not appear to be an adaptive enzyme since it is found in plants cultured aseptically in solutions free from combined nitrogen. The activity of this enzyme seems to depend upon an intact particulate structure; all attempts to obtain a soluble preparation resulted in a loss of activity.

It has often been suggested that the reduction of nitrate in green plants

is coupled with photochemical reactions. The metabolic relations of the reduction of nitrate and nitrite with special reference to photosynthesis have been explored in green algae by Kessler (80, 81). In experiments with *Ankistrodesmus braunii* he studied the reduction of nitrate and nitrite under various conditions, including light and dark and in presence and absence of CO_2. The data lead to the conclusion that in this alga the reduction of nitrate is only indirectly linked to photosynthesis as a result of the synthesis of carbon compounds. Nitrite reduction, on the other hand, appears to be closely coupled to the photochemical reactions by means of hydrogen carriers or phosphorylation.

There is, however, much evidence that both nitrate and nitrite can undergo rapid transformation in root tissues and here the marked changes of respiratory rate and quotient during their assimilation [Willis & Yemm, (181)] indicate a close coupling with cell respiration. Much of the progress which has been made in clarifying the enzymic mechanisms associated with nitrate reduction has again emphasized their close relation with pyridine nucleotides and other oxidation-reduction systems which operate in cell respiration or photosynthesis.

The assimilation of nitrate and that of ammonia are somewhat differently influenced by physiological conditions. This may be in part owing to differential effects of pH on the uptake of nitrate or ammonium ions and to interactions with the supply of other essential mineral nutrients as disclosed by the experiments of Street (154) with root tissue cultures. Data collected by MacMillan (97) indicate that ammonia is taken up by some fungi mainly in an undissociated form. Green algae vary considerably in their ability to assimilate nitrate and ammonia. In a survey of six species Proctor (124) has shown that some, such as *Chlamydomonas* and *Chlorella*, take up NH_4^+ from culture solutions containing NH_4NO_3 until only NO_3^- remains; others (*Pandorina* and *Haematococcus*) take up NO_3^- much more rapidly than NH_4^+. Syrett (157, 158) examined the problem in some detail in *Chlorella*. Cultures, grown for a preliminary period in the light in solutions deficient in nitrogen, had a much higher capacity for assimilating NH_4^+ than NO_3^- in the dark. The difference was reflected in a greater accumulation of soluble nitrogenous constituents, accompanied by depletion of carbohydrates in the cultures supplied with ammonia. Syrett suggested that the limited power of nitrate assimilation is due to the failure to reduce nitrate when the carbohydrate content of the cell falls. It seems probable that in such algae the reduction may be brought about more readily by hydrogen donors generated in photochemical reactions.

Primary synthesis of amino acids.—The central position of ammonia in the nitrogen metabolism of plants is now generally acknowledged. The rapidity with which ammonia can be assimilated by many plants, and the demonstration that it is readily formed by enzymic reduction of nitrate and during nitrogen fixation has given support to this view. But from time to

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time other pathways of assimilation have been visualized. Hydroxylamine, a probable intermediate in nitrate reduction, has been regarded as a starting point for the biosynthesis of amino acids, either by way of oximes, or by conversion of pyridoxal to pyridoxamine as a preliminary step in transamination reactions. The possibility of hydroxylamine acting as an intermediate in amino acid synthesis has been considered in earlier reviews in this series (149, 183), but little critical evidence can be advanced to sustain it. The many ways in which pyridoxine can take part in cellular metabolism offer alternative explanations to that put forward by Silver & McElroy (138) to account for the requirement of pyridoxine when mutant strains of *Neurospora* are grown with sources of nitrogen other than ammonia. A serious difficulty which has confronted experimental work in this field is the high toxicity of hydroxylamine when supplied externally to plant tissues. In the limited assimilation of hydroxylamine by excised barley roots, Yemm & Willis (189) found no evidence of a distinctive pathway of amino acid synthesis; the products formed in the roots were similar to those when ammonia or nitrate were supplied although much smaller in quantity.

The assimilation of ammonia by plants continues to occupy much attention. There are now many examples, including higher plants and microorganisms, in which a conspicuous increase of glutamic acid and glutamine has been observed when nitrate or ammonia is assimilated (111, 130, 135, 171, 188, 189). Strong evidence has been gained by isotopic labelling to support earlier suggestions that glutamic acid is one of the most important products when ammonia and indeed when nitrite, nitrate, or gaseous nitrogen are assimilated.

The rapid labelling by N^{15} of the dicarboxylic amino acids, glutamic and aspartic, and their amides glutamine and asparagine, was indicated by earlier experiments of Vickery *et al.* (170), MacVicar & Burris (99), and Yemm & Willis (189). A more detailed study has recently been carried out by Cocking (31) of the assimilation of N^{15}-ammonium phosphate by young seedlings of barley. Roots from representative samples of the seedlings were analysed after short periods of assimilation; by chromatography on ion-exchange resin it was possible to separate the chief free and combined amino acids in order to investigate the abundance of N^{15} in them. The results showed uniformly that the highest labelling occurred in the free glutamic acid and glutamine of the roots. Particularly after short periods (1 to 2 hrs.) the abundance in glutamic acid and glutamine was much greater than that in aspartic acid. With the size of the metabolic pool of free glutamic acid and glutamine taken into account, a rapid primary synthesis and turnover of these metabolites during the assimilation of ammonia was demonstrated. A close parallel to these changes in intact roots was found in food yeast by Sims (140) who developed more sensitive and satisfactory methods for measurement of N^{15} abundance in amino acids. In yeast cultures in a steady state of exponential growth, unequivocal evidence was obtained

of a rapid synthesis of glutamic acid; within a few minutes of supplying N^{15}-ammonium phosphate, glutamate was labelled much more heavily than any other amino acid. From the rate of turnover of the amino group of the glutamate, it was possible to estimate that over 90 per cent of the nitrogen assimilated by the yeast could be accounted for by this route.

Further evidence has been found of the wide distribution in plants of enzymic mechanisms for the synthesis of glutamic acid from ammonia and α-ketoglutarate. The preparation and chief characteristics of glutamic acid dehydrogenase from corn leaves have been described by Bulen (20). The enzyme is specific for DPN, and its action does not appear to depend upon trace metals since it is not inactivated by dialysis against cyanide solution. Fincham (48, 49) has partially purified glutamic acid dehydrogenase from *Neurospora* and has studied its activity in mutants. The mutant strains which appear to lack the enzyme have a requirement for one of a number of amino acids for normal growth. Dehydrogenases acting upon amino acids other than glutamic acid have not been detected in plant tissues although Wiame & Piérard (180) and Fairhurst *et al.* (46) obtained from *Bacillus subtilis* an alanine dehydrogenase which synthesized alanine from pyruvate and ammonia.

Investigations of the assimilation of nitrate and nitrite by roots and by yeasts have yielded no evidence that distinctive mechanisms of amino acid synthesis are brought into action when nitrogen is supplied in these forms. For example, in excised barley roots, Yemm & Willis (189) found that nitrite gave rise to an accumulation of glutamine as great as or greater than that from ammonia. With food yeast Sims (140) has shown that the metabolic pool of free amino acids during exponential growth of cultures on nitrate media did not differ substantially from that when ammonium salts were supplied. In particular no increase of aspartic acid or asparagine occurred in nitrate-fed yeast, such as would be expected if an additional mechanism of synthesis of aspartic acid operated by way of hydroxylamine and oximinosuccinic acid (171). The data are consistent with the view that nitrate is reduced to ammonia prior to formation of amino acids, but critical experiments with labelled nitrate are required. It may be concluded that studies both of the assimilation of inorganic nitrogen by plant cells and of their enzymic properties have further strengthened the position of glutamic acid as the chief primary product in the synthesis of amino acids.

Interconversion of Amino Acids

Further metabolism of the primary products of nitrogen assimilation is generally considered to involve extensive interconversion of amino acids, giving rise to the wide array of amino acids commonly found in plant tissues. Such interconversions may be broadly classified into two types: those involving the transfer of the amino group only (transamination reactions), the carbon skeleton of the newly formed amino acid being derived else-

where; and those in which the whole amino acid undergoes reaction with modification of its carbon skeleton. In a recent and comprehensive monograph Meister (110) has considered both the processes of transamination and interconversion of the carbon skeletons of amino acids. These topics have also been fully covered by the earlier reviews of Meister (109) and of Davis (40), and in the McCollum-Pratt symposium. *Amino Acid Metabolism* (94). In the following paragraphs only more recent work will be considered.

Transamination.—The wide scope of transamination is apparent from Meister's monograph (110) in which more than 60 transamination reactions are listed. A recent investigation extends this list still further: Sallach (136) has extracted from mammalian tissues a transaminase which will bring about the formation of hydroxyaspartic acid from dihydroxyfumaric acid and glutamic acid.

The specificity of the transaminases is still far from known, but recent studies of Fincham & Boulter (50) indicate an interesting approach to the problem. They studied the effects of the amino acid composition of the culture medium on the production of transaminases in *Neurospora crassa* and found that in a minimal medium transaminases for aspartic acid, alanine, and ornithine only could be detected. Feeding with any one of several amino acids led to an increased production of ornithine transaminase and very large increases in nine other transaminase activities, all of which maintained constant proportions to one another which were independent of cultural conditions. Fincham & Boulter suggested that the nine amino acids, phenylalanine, isoleucine, valine, leucine, norleucine, norvaline, methionine, γ-aminobutyric acid, and tryptophan were all transaminated by a single enzymic protein.

In view of the rôle of vitamin B_6 in the transamination reaction, it is not surprising that several workers have noted increased transaminase activity in cell-free extracts when pyridoxal phosphate or pyridoxamine is added. Among these are Bigger-Gehring (11) who studied the range of transamination reactions in *Saccharomyces fragilis*. Martos (104) reported that decotylised pea seedlings were deficient in glutamic acid and also low in pyridoxine as compared with intact seedlings; infiltration of pyridoxine led to increased levels of glutamic acid in decotylised seedlings. From evidence of a pyridoxamine requirement in lactic acid bacteria when tyrosine or phenylalanine of the culture medium was replaced by the corresponding keto acid, Holden (70) inferred that these two amino acids originated from transamination reactions in these organisms.

While many transamination reactions have now been demonstrated conclusively, there is still room for speculation as to their extent and metabolic importance in higher plants. It is for this reason that the list of keto acids known to occur in plants which was given by Steward & Pollard (149) is particularly valuable; it indicates clearly that the keto acid substrates for possible transamination reactions are widely available in higher plants.

Interconversions of carbon skeletons.—In the study of the metabolic conversions of amino acids involving chemical changes of the carbon skeletons, three main lines of evidence may be distinguished. Until recently probably the most rewarding of these lines has been the study of the genetics and nutrition of various biochemical mutants, chiefly of microorganisms such as *Neurospora.* Work in this field has been recently reviewed by DeBusk (42). With the wide availability of isotopically labelled metabolites, much progress has recently been made by tracing the pathways of metabolism, especially in microorganisms. Although difficulties of interpretation of isotope experiments often occur, there is little doubt as to the power of the tracer technique when used with understanding. Complete confirmation of the metabolic patterns inferred from the use of mutants or of isotopes must, however, generally await the isolation of the appropriate enzyme systems from the organism in question; it is only when this type of evidence reinforces and is reinforced by evidence from one of the other two sources that the occurrence and nature of a cellular reaction may be said to be established.

In recent years a striking feature of the study of amino acid metabolism has been the recognition of "families" of closely related amino acids. These families and their chief members are as follows: glutamic family—glutamic acid, proline, and arginine; aspartic family—aspartic acid, methionine, threonine, isoleucine (diaminopimelic acid and lysine in some organisms); serine family—serine, glycine, and cysteine; pyruvic family—alanine, valine, and leucine; aromatic family—tryptophan, tyrosine, and phenylalanine. The existence of these families is perhaps best illustrated by the extensive studies of the Carnegie Institution group. Roberts *et al.* (133) investigated biosynthesis in *Escherichia coli* and, less extensively, in *Neurospora, Torulopsis,* and *Chlorella.* The method generally employed was to study the competitive effects of various amino acids upon the incorporation of C^{14} from glucose or acetate into the amino acids of the proteins. Thus, for example, glutamic acid was able to inhibit not only C^{14} incorporations into the glutamic acid of the cellular proteins but also into proline and arginine as well, whereas ornithine or citrulline inhibited incorporation into arginine only. A somewhat different method was used with *Torulopsis utilis* by Cowie & Walton (35). They used log phase yeast and after adding C^{14}-fructose followed the increase of radioactivity both in the soluble fractions and in the protein. They again found evidence of family relationships since, of the soluble amino acids, the heads of the families became labelled first when the organism was supplied with C^{14}-fructose and these same amino acids were the first to lose radioactivity when the yeast was transferred back to C^{12}-fructose. When this happened the other members of the family at first increased in C^{14} content at the expense of the head amino acid, thus providing further evidence of their secondary origin.

Among other recent studies employing isotopes, the following appear to extend our knowledge of amino acid metabolism in microorganisms and

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animals. Vogel (174) has shown that the carbon of ornithine may be incorporated not only in arginine but also into proline and glutamic acid when supplied in low concentrations to *E. coli*. This indicates that the accepted glutamate⟶ornithine pathway may be reversible. Krasna *et al.* (84) have demonstrated that the N^{15} of labelled threonine is incorporated by *Streptomyces griseus* very largely into the aminopropanol moiety of vitamin B_{12}. This suggests that threonine may be decarboxylated in this organism. After adding glycine-2-C^{14} to growing mycelium of *Fusarium lycopersici*, Kern *et al.* (78) found C^{14} not only in glycine and serine of the protein but also in large amounts in valine and leucine, thus indicating a possible extension of the serine family in some fungi. A possible alternative route of arginine synthesis in mammalian tissues is suggested by observations of Horner *et al.* (71) on the synthesis of arginine from guanidoacetic acid; kidney homogenates from rat gave significant incorporations of C^{14} from guanidoacetic acid into the amidine carbon of arginine.

A convincing combination of genetic and enzymic studies has been described by Yanofsky (184) in support of current theories of the synthesis of tryptophan. He showed that each member of a series of tryptophan autotrophs lacked one or more of the enzymes necessary for the full reaction sequence from anthranilic acid to tryptophan. Other recent enzymic studies include the preparation from pea seedlings by Clark & Mann (29) of an amine oxidase oxidizing tryptamine to 3-indoleacetaldehyde, a possible step on the route from tryptophan to indoleacetic acid, and the demonstration by Wolff *et al.* (182) that an enzyme from yeast can utilize methyl mercaptan and L-serine for the synthesis of S-methylcysteine.

Amino acid interconversions in higher plants.—From higher plants there is far less direct evidence for amino acid interconversions than that from microorganisms which has already been discussed. It has, however, been established by Folkes & Yemm (52) that the changes which take place in the amino acid composition of barley grains when they germinate may be adequately explained in terms of amino acid interconversion reactions such as commonly occur in microorganisms. It therefore seems more profitable to discuss the amino acid metabolism of higher plants in terms of such reactions, supported as they are by comparative biochemistry, rather than in terms of ill defined ideas like the alternative hypothesis. Folkes & Yemm's experiments employed seedlings of barley grown without external supplies of nitrogen; samples were taken at intervals for analysis of the chief protein and non-protein nitrogenous constituents. From the results and analyses of the amino acid composition of the proteins of barley [Folkes & Yemm (51)], it was possible to calculate the total amount of each amino acid in the seedlings at various stages of germination. A striking feature of the results was that the endosperm contained in its protein reserves over 70 per cent of the amino acid residues required by the developing embryo. Additional requirements of the embryo included amino acids, such as aspartic

acid, glycine, lysine, and arginine, as well as chlorophyll and nitrogen bases; these were synthesized from excess amino acids of the endosperm such as glutamine and proline. The close parallel of this situation with that operating in tissues assimilating inorganic nitrogen is clear and suggests that the synthesis of amino acids from the primary products of nitrogen assimilation involves similar reactions to the interconversion processes in germinating barley. Glutamine is clearly central in these reactions, but Folkes & Yemm obtained different results when they attempted to estimate the level of free glutamine in the embryos by indirect methods and by the quantitative paper chromatography of Thompson & Steward (163). These differences have since been resolved by Sampaio & Folkes (137) using ion-exchange chromatography. They pointed out that the incomplete separation of glutamine and alanine on filter paper may give misleading results; the free glutamine of the embryo is in fact relatively low, far more glutamic acid and amide residues being associated with the peptide fraction than with the free amide. An even more striking unbalance between "free" and "peptide" was apparent for some of the amino acids, notably proline, methionine, histidine, and arginine which were plentiful in the peptide fraction but undetectable in the free state at some stages of germination. These results suggest a special importance for the peptide fraction and will be further discussed later in this review.

Some of the metabolic features of seedlings are paralleled in developing seeds; the nitrogen metabolism of developing pea seeds has been the subject of three recent investigations. In these studies Raacke (125, 126, 127) used only indirect methods of analysis, but McKee *et al.* (96) employed qualitative paper chromatography, and Spragg (145) quantitative paper chromatography, to estimate the various amino acids during seed development. Spragg extracted free amino acids in 75 per cent ethanol and then used hot water to extract peptides which he also analysed. While such extraction procedures may be open to criticism on the grounds that they do not reliably separate free amino acids from peptides, the overall picture given by Spragg's results appears to be consistent. He found a considerable accumulation of free amino acids and peptides within the immature pea seed. These metabolites reached a maximum by about 18 to 21 days after flowering; a rapid fall subsequently was associated with the initiation of synthesis of the storage globulins, vicilin, and legumin. Conspicuous in the 75 per cent ethanol extract were glutamine and arginine; together they contained about half of the total nitrogen of the extract. Somewhat similar results were obtained by McKee *et al.* (96).

The importance of arginine metabolism in the developing pea is paralleled in several other plant organs. Oland & Yemm (120) reported arginine as a major constituent of the soluble nitrogenous reserves of apple stems, accounting for over 60 per cent of the nitrogen soluble in 70 per cent ethanol. It is noteworthy that they found arginine to be incompletely extracted by

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this solvent but removable by hot water or salt solutions. This fact does not appear to be sufficiently realized and may lead to difficulties in apparent separations of amino acids and peptides (cf. comments on Spragg's work above). These findings may also in part account for the very high arginine content of the alcohol-insoluble residue of tulip bulbs analysed by Zacharius *et al.* (190). This "bulk protein," as they called it, contained 47.7 per cent of its nitrogen in the form of arginine. Whatever the interpretation of this component, there is little doubt that arginine is of major metabolic importance in the tulip since it is also the most conspicuous free amino acid of the bulb and is apparently mobilized during floral development; only after this do glutamine and asparagine become predominant.

The two amides were found by Bollard (13) to be generally the most plentiful nitrogenous compounds in the xylem sap of many plants. The importance of the amides as translocatory products has long been suspected, but Bollard's direct demonstration is nevertheless welcome. His observations that nitrate is present only in traces in the sap and that nitrogen is translocated largely in organic form are also important and indicate that protein synthesis in most organs depends upon the secondary metabolism of amino acids rather than upon the primary assimilation of inorganic nitrogen. The synthesis of glutamine and asparagine in roots supplied with inorganic nitrogen [Yemm & Willis (189), Cocking (31)] is consistent with this view. Glutamine and asparagine have also been found by Mansford & Raper (103) to increase prior to spore formation in *Equisetum*.

The investigations reviewed above confirm the importance of the amides and of arginine as the chief compounds involved in the mobilization and transfer of nitrogen. In this connexion the observations of Harris (65) on the utilization by isolated oat embryos of various amino acids are of interest. Of 12 amino acids tested, only arginine was not inhibitory to root growth when used as the sole source of nitrogen. Other amino acids needed to be supplied in balanced mixtures, otherwise antagonistic effects could be demonstrated. It is unfortunate that Harris does not appear to have tried glutamine as a nitrogen source since Rijven (132) found this amide to give good growth of the embryos of 12 different species that he investigated. He was able to demonstrate a normal Michaelis-Menten relationship between glutamine concentration and growth but found asparagine to be inhibitory at levels above about 10 mg./l. Effects of nitrogen nutrition on growth rates have also been studied by Almestrand (4) using isolated cereal roots.

Several investigators have studied the effects of abnormal mineral nutrition upon nitrogen metabolism. In a series of researches Thompson & Morris (161, 162) have followed changes in the amino acid composition of turnips or other plants brought about by mineral deficiency or by wilting. Increased levels of glutamine were associated with deficiencies of potassium, phosphorus, and sulphur, but nitrogen deficiency led to low glutamine levels. Similar effects with micronutrients were observed by Possingham (122),

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increased levels of all amino acids being found with deficiencies of copper, zinc, manganese, and iron, the last also inducing high amide contents. Molybdenum deficiency had the reverse effect, and increased amino acid levels could be observed within a few hours of supplying molybdenum to deficient plants (123). Full understanding of the rather complex effects of mineral nutrition must await advances in our knowledge of the enzymic processes involved. The suggestion by Coleman & Hegarty (33) that the occurrence of putrescine in potassium-deficient barley is due to effects on ornithine decarboxylase is of interest in this connexion.

Metabolism of nonprotein amino acids.—Of the amino acids not known to be constituents of proteins, γ-aminobutyric acid has aroused most interest. For example, in their experiments on carrot tissue supplied with C^{14}-labelled glutamine and γ-aminobutyric acid, Steward *et al.* (148) showed that, while γ-aminobutyric acid could act as a source of labelled glutamic acid, little or no label was found in γ-aminobutyric acid when glutamine was the labelled substrate. From this they concluded that the carboxylation of γ-aminobutyric acid might represent an important pathway for the synthesis of glutamic acid. Similar suggestions have been made by Baptist (9) and by Mansford & Raper (103) who have demonstrated the occurrence of the γ-amino acid in a wide range of plants. However, it seems that carrot tissue may not be typical in this respect; in many plants γ-aminobutyric acid appears to be formed chiefly by decarboxylation of glutamic acid. The most direct evidence for this view is that obtained by Cocking (31) from analyses of the roots of barley seedlings grown in water culture and supplied for a short period with N^{15}-ammonium phosphate. He found that while glutamic acid was rapidly and heavily labelled the γ-aminobutyric acid showed consistently lower isotopic abundances. It is probable that accumulation of the γ-amino acid may be associated in some tissues with oxygen limitation; Sims (140) could detect the acid only in very small amounts in rapidly growing log phase cells of *Torulopsis utilis* but found that when protein synthesis was checked by partial anaerobiosis, glutamic acid accumulated and γ-aminobutyric acid was then plentiful. Similarly, Naylor & Tolbert (115) found that the greatest labelling of γ-aminobutyric acid in barley leaves supplied with C^{14}-glutamic acid occurred when the leaves were maintained in the absence of oxygen; under aerobic conditions the majority of the label in the soluble fractions was located in glutamic acid and glutamine. Further experiments of the type described by Steward *et al.* (148) and by Cocking (31) are obviously necessary before the metabolic position of this amino acid is fully resolved, but it seems that besides differences in the metabolic state of various plants and organs differences in enzymic complement may also be important. For example, Kulkarni & Sohonie (85) have recently shown large variations in glutamic decarboxylase activity within a single family of flowering plants, the Leguminosae.

Only brief mention need be made of other amino acids. β-Alanine has

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been found by Mansford & Raper (103) to be present only in the higher plants that they investigated and not in horsetails and nonvascular plants; according to Champigny & Lioret (25) this acid in higher plants is largely confined to the "peptide" fraction, possibly in the form of coenzyme A. Ornithine and citrulline have been found to be conspicuous in the free amino acids of log phase *Torulopsis* (140) and citrulline is notable in the moss *Funaria* (103). Rabson & Tolbert (128) have shown that excised roots of Alaska peas will convert 15 per cent of absorbed aspartic-2,3-C^{14} acid to homoserine in three hours in the dark. Virtanen & Ettala (172) report a new amino acid, dihydroxyglutamic acid, to be widely distributed in the stems and leaves of higher plants, and in ferns, mosses, and Basidiomycetes. The cyclic amino acid, 1-aminocyclopropane-1-carboxylic acid, recently reported by Burroughs (23) to occur in perry pears has now been found in cowberries by Vähätalo & Virtanen (168). The occurrence of many unusual amino acids has been most usefully surveyed by Steward & Pollard (149); the metabolic importance of many of these compounds is still a matter of doubt, but it is a striking feature of the majority that their metabolic activity appears to be low.

Amino Acids and the Metabolism of Proteins and Peptides

The basic problems of the participation of amino acids in the synthesis of proteins are being attacked over a broad front. The incorporation of labeled amino acids into protein has been studied both in intact cells and in cell-free preparations; some further light has thus been thrown on the complex relationships between the metabolic pool of free amino acids and the synthesis of protein. In a somewhat different approach, enzymic mechanisms which catalyse carboxyl activation of amino acids by ATP have been found widely distributed in animals, plants, and microorganisms although little further knowledge has been gained concerning the significance of enzymic transamidations and transpeptidations in cell metabolism. Because of their possible intervention in protein synthesis, the peptide constituents of some plant tissues have been more thoroughly examined, while the close relation between the metabolism of proteins and nucleic acids continues to focus attention on the problems of reduplication during growth and reproduction.

Utilization of amino acids in protein synthesis.—Since the experiments of Brachet (18) on the assimilation of C^{14}-glycine by *Acetabularia* much additional evidence has been obtained by means of labelled amino acids. Stephenson *et al.* (146) in experiments with leaf discs of tobacco obtained strong evidence that labelled amino acids are incorporated into proteins in intact cells. Using leucine, valine, alanine, or glycine containing C^{14} in the carboxyl group, a steady increase in radioactivity in various fractions of cell proteins was observed by methods which seem to preclude the retention of amino acids in nonpeptide linkages or complications due to the activity

of microorganisms. The incorporation was greater in the light than in the dark, and under aerobic than under anaerobic conditions, indicating a close coupling with energy-yielding processes. It is unfortunate that only the total radioactivity in the protein was measured, so that the retention of labelling in individual amino acids supplied exogenously to the tissue remains uncertain.

A study of the labelling of proteins in wheat leaves supplied with C^{14}-glutamine or C^{14}-glucose has been made by Bidwell *et al.* (10). In light substantial labelling of glutamic acid in the protein from C^{14}-glutamine was observed; a comparison of the experiments with glucose and glutamine indicates that labelling of glutamic and aspartic acid in the protein reflects fairly closely that of the free amino acids. A different distribution of labelling was found in leaves in the dark; it is suggested that the entry of glucose into the tricarboxylic acid cycle is inhibited in the light. A more detailed analysis of these results is not possible since no account is given of the quantitative changes of free amino acids and the changes of specific activity. The experimental data of a study of the utilization of C^{14}-glucose, C^{14}-glutamine, and C^{14}-γ-aminobutyric acid by tissue cultures of carrot have been described by Steward *et al.* (148). Steward & Bidwell (147) conclude, chiefly from a consideration of the labelling of the amino acids in the metabolic pool and in the proteins, that the data support the alternative hypothesis of protein synthesis in which the carbon skeletons are derived directly from carbohydrate and the nitrogen from effective donors such as glutamic acid and glutamine, operating in a continuous protein cycle in which much of the respiratory CO_2 originates from protein metabolism. However, the evidence to support this concept of protein synthesis is by no means decisive. As will be discussed more fully later in this review, special features of the organization of vacuolated plant cells in which a large part of the free amino acids may be relatively inaccessible to the centres of protein synthesis could readily account for many of the distinctive features in the labelling of free and combined amino acids in the tissues.

Some of the most complete and direct evidence in support of the amino acid hypothesis of protein synthesis has been obtained in a comprehensive study of biosynthesis in *E. coli* by Roberts *et al.* (133). Much evidence is given that amino acids are precursors in protein synthesis, and the hypothesis is developed that amino acids are bound by macromolecular constituents of the cell as a primary step in protein formation. The ease with which amino acids, supplied exogenously, equilibrate with cell constituents clearly indicates a relatively simple organization in the bacterial cells. More limited observations were recorded with cultures of *Torulopsis, Neurospora* and *Chlorella;* with some differences in detail a similar pattern of amino acid metabolism underlying protein synthesis was demonstrated. Cowie & Walton (35) carried out similar experiments with *Torulopsis* in which competition of free amino acids with isotopic labelling of proteins from C^{14}-fructose

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was examined. In this yeast there was evidence of a substantial metabolic pool of amino acids which complicated the effects of isotopic competition. Detailed analysis of exponentially growing yeast cells by Sims (140) has shown the presence of a varied pool of amino acids containing nearly all of those which occur naturally in proteins and including relatively large quantities of glutamic acid and glutamine.

Much evidence consistent with the amino acid hypothesis of protein biosynthesis has been gained by experiments in which isotopic nitrogen has been used to trace the course of assimilation in plants. Cocking (31) found that when young seedlings of barley were supplied with labelled ammonium salts, enrichment with N^{15} was detectable in many of the free amino acids of the root tissues within a few hours. Subsequently the labelling of the amino acids combined in the tissue proteins increased as would be expected if the free amino acids were directly involved in protein synthesis. Similar results have been obtained with food yeast by Sims (140), and by Burris and his collaborators (3, 21) in their investigations of nitrogen fixation and ammonium assimilation by *Azotobacter*. The results of Turba *et al.* (167) using carbon-labelled substrates lead to the same conclusions; when C^{14}-acetate was supplied to log phase *Torulopsis* the individual free amino acids became rapidly labelled but there was a well-marked induction phase before the amino acid residues of the proteins were labelled. Such observations are difficult to reconcile with the alternative hypothesis.

It is clear, however, that the incorporation of labelled amino acids into tissue proteins is not decisive evidence of their participation in the normal processes whereby new proteins are synthesized. Work with cell-free preparations from both plants (175) and microorganisms (56) indicates that exchange reactions, different in several important respects from the processes of the complete synthesis of protein, may bring about incorporation of amino acids into proteins. Gale (56) has recently discussed much of the evidence bearing on this problem. With plant tissues such as excised leaves and roots, it has often been found that isotopically labelled metabolites are incorporated into tissue proteins under conditions in which no net synthesis of protein is detectable [Chibnall & Wiltshire (28), Yemm (187), Yemm & Willis (189)]. Such observations have sometimes been interpreted as evidence of a continuous protein cycle, operating by independent processes of breakdown and synthesis of protein. The possibility that exchange reactions of much more limited scope are responsible for the dynamic state of cell proteins is now clearly indicated.

The formation of adaptive enzymes has provided a sensitive method for detecting the synthesis of new proteins of specific structure. In this way the relation between amino acid metabolism and protein synthesis has been studied particularly in yeasts and microorganisms. In a general review of the formation of adaptive enzymes, Spiegelman (144) has marshalled evidence which supports the view that free amino acids are directly involved

in the synthesis of new enzymic proteins. In yeast Halvorson and others (60, 62) have shown that induced synthesis of maltase is decisively influenced by depletion of the pool of free amino acids and is inhibited by tryptophan analogues which also inhibit growth and the utilization of the pool of free amino acids. Slonimski (142) investigated the adaptive formation of respiratory enzymes in yeast in relation to amino acid metabolism and demonstrated the inhibitory action of an amino acid analogue, *p*-fluorophenylalanine. A rapid incorporation of glycine-2-C^{14} into cytochrome was observed by Yčas & Drabkin (186) in yeast during its adaptation to aerobic conditions. In bacteria, notably *E. coli,* other evidence such as that of Løvtrup (91, 92) has been gained of the dependence of the formation of adaptive enzymes on the synthesis of proteins from amino acids.

Enzymic mechanisms for the synthesis of peptide bonds.—The possibility of studying the biosynthesis of peptide bonds in cell-free preparations has attracted much attention. The rôle of ATP is now well established not only in the synthesis of amides and some simple peptides but also in the incorporation of amino acids into subcellular structures such as those studied by Webster (176, 177) and Gale (56). More recently the widespread occurrence of enzymes which promote the carboxyl activation of amino acids by ATP has been indicated in preparations made from animals, microorganisms, and plants.

Hoagland and his collaborators (67, 68) separated a soluble enzyme from animal tissues which catalysed the exchange of P^{32}-pyrophosphate with ATP in the presence of amino acids and which promoted the synthesis of hydroxamates from hydroxylamine and twelve different amino acids. Since a free AMP-amino acid compound could not be detected, it was suggested that the general reaction could be formulated as follows:

$$\text{Enz.} + \text{ATP} + \text{Amino acid} \rightleftarrows \text{Enz.}\begin{matrix}\diagup\,\text{AMP}\\ \diagdown\,\text{Amino acid}\end{matrix} + \text{pyrophosphate.}$$

In this system hydroxylamine may act as a trapping agent to form hydroxamates. Later Keller & Zamecnik (77) suggested that carboxyl activation of amino acids is a preliminary step in protein synthesis and showed that another nucleotide, guanosine diphosphate or triphosphate, while not replacing ATP, was involved in amino acid incorporation in cell-free preparations from animal tissues. The activation reaction with different amino acids indicated the presence of a number of enzymes differing in their specificity; Cole *et al.* (32) have effected a partial purification and separation of enzymes activating differen groups of amino acids.

The wide distribution of similar enzymes in microorganisms, including *Neurospora* and yeast, was shown by DeMoss & Novelli (43). Davis *et al.* (41) and Clark (30) have briefly reported the presence of amino acid

activating enzymes in the soluble fraction of cell-free preparations from a wide variety of higher plants. On the other hand Webster (179), working with preparations from pea seedlings, found that these enzymes were associated with RNA-protein particles which incorporated C^{14}-amino acids. The action of inhibitors and fractionation of the preparations led him to propose that incorporation involves a sequence of at least three reactions in which activated amino acids were transferred by carboxyl linkage to an intermediate compound and then took part in peptide bond formation. Hoagland *et al.* (69) have indicated that intermediate compounds between amino acids and RNA of low molecular weight may be important in protein synthesis, and Hansen & Hageman (64) have isolated glutamic and aspartic acid derivatives of ADP from animal tissue. As briefly discussed by Crick (36), it is possible that nucleotides act as specific adaptors in the orientation of amino acids on RNA.

The potentialities of animal and plant proteinases for building up specific peptide structures by transamidation and transpeptidation was discussed by Hanes *et al.* (63), Fruton (55), and others. In this connexion several investigations of the activity of papain have been described. In experiments with a highly purified preparation, Johnson & Herriott (74) have shown that acylated peptides, notably glutamyl dipeptides and glutathione, promote the breakdown of glycinamide and some dipeptides; there was no evidence that transpeptidation was involved. The possibility that interaction between peptides is important in regulating the synthesis of polypeptides in papain-catalysed reactions with glycinamide has been indicated by Tollin & Fox (164).

Although transpeptidation occurs in the somewhat special case of plastein formation [Haurowitz (66)], there is no clear evidence that such reactions are important in cell metabolism. It is unfortunate that investigations of proteinase activity in plant tissues are generally concerned only with hydrolytic action. Robinson (134) has shown that well marked changes in proteolytic activity occurred during the development and differentiation of meristematic cells of broad bean and has suggested that these changes may regulate the balance between synthesis and breakdown of proteins during growth. The possibility that inhibitors of proteinases are important in this repect was indicated by the data of Ambe & Sohonie (5).

Occurrence and metabolism of peptides.—In many classical studies of plant metabolism the existence of a peptide fraction has been recognized, usually based upon indirect methods of analysis. Peptides have generally been estimated from the increase of amino nitrogen upon hydrolysis of a plant extract which has been freed from protein by heat coagulation or by dialysis. Such indirect methods have been employed in two recent investigations. Folkes & Yemm (52) found in germinating barley, even under conditions of nitrogen deficiency, the peptide fraction accounts for 5 to 10 per cent of the total nitrogen of the seedling. In developing peas Raacke (125,

126) showed that peptides accumulate in the seedcoats and cotyledons prior to synthesis of protein in the embryo. Similar observations have been made by Spragg (145) who estimated peptide, after removal of protein by heat coagulation, from the aqueous extract made after extraction with 75 per cent ethanol (see previous comment, p. 255). He showed the fraction to be commensurate with the difference between the total dialysable nitrogen and the ethanol-soluble nitrogen and so presumed that the whole of his peptide fraction was dialysable and consequently had a maximum molecular weight of about 10,000. Upon paper chromatography of this fraction Spragg was able to detect up to five discrete slow-running spots which changed in relative amounts during seed development; these spots were presumed to represent peptides. Amino acid analyses of the fraction indicated the presence of at least 12 different amino acids: a striking feature was the high level of arginine-nitrogen, representing between 30 and 50 per cent of the total nitrogen of the fraction, and also the fact that apparently all of the dicarboxylic acid residues of the peptides were amidated. A similar one-to-one relationship between amide nitrogen and dicarboxylic acid nitrogen was found for the peptides of the barley embryo by Folkes & Yemm (52), while Thomas & Krauss (160) report that peptides rich in basic nitrogen (arginine?) accumulate in *Scenedesmus* when supplied with nitrate after a period of nitrogen deficiency.

While chief interest in the metabolism of peptides has centered upon their possible position as intermediates in protein synthesis, other metabolic rôles have also been assigned to them. Raacke (127) has recently suggested that they may be involved also in the translocation of nitrogen: the accumulation of peptides in the developing pea seed is associated with a depletion of the peptides of the pod. Similar suggestions have been made by Sampaio & Folkes (137) from their study of the amino acid composition of peptide fractions of germinating barley. They found that in the endosperm the overall composition of the peptides reflected closely the amino acid content of the storage proteins which were being mobilized, an indication that the peptides were probably derived directly by partial hydrolysis of the endosperm proteins. In the embryo the peptide fraction which was relatively rich in glutamic acid showed little similarity in composition either to the free amino acids or to the protoplasmic proteins and consequently did not appear to represent a direct intermediate in protein synthesis. On the other hand, it did resemble closely in amino acid composition the peptides of the endosperm at the same stage of germination and this strongly suggested that translocation of peptides might take place. The position is, however, far from simple because only about 50 per cent of the embryo peptide fraction was freely diffusible and hence it is improbable that all of the fraction could have been derived directly by translocation. Sampaio & Folkes point to the fact that peptide accounts for up to 10 per cent of the nitrogen of N-deficient barley as an indication of the probable metabolic importance of the fraction.

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While such considerations may sometimes be useful, they are nevertheless dangerous; Morton & Broadbent (114) have shown for several fungi that up to 35 per cent of the assimilated nitrogen may be excreted in the form of peptides which are not then available for further metabolism.

More direct evidence on the metabolism of peptides has been obtained by Turba & Esser (165) who used log phase yeast (*T. utilis*) supplied with glucose, ammonium salts, and C^{14}-acetate. From the yeast they extracted a series of alcohol-soluble peptides and separated them by high tension ionophoresis; in all they isolated about 40 acidic peptides which were rich in glutamic acid. These peptides became rapidly labelled with radiocarbon, the curves of incorporation for amino acids, peptides, and proteins lying parallel and in that order so that the peptides were more highly labelled than the proteins for the first 60 minutes of exposure to C^{14}-acetate. Essentially similar results have been obtained for *Pseudomonas hydrophila* supplied with C^{14}-glucose by Connell & Watson (34). They isolated 11 distinct peptides each containing 5 to 11 amino acids. While such evidence may be held to favour the participation of peptides in protein synthesis, the relationships demonstrated could be fortuitous and may result only from a parallel labelling of the peptide and protein pathways. To decide between these two hypotheses, it would be necessary to obtain evidence of metabolic turnover of the peptides commensurate with incorporation in the proteins. In the somewhat special case of glutathione Turba *et al.* (166) have shown, from the sigmoid nature of the curve of incorporation of C^{14} and S^{35} into this tripeptide, that a rapid turnover occurred, the half life of glutathione being about 22 minutes. It is possible that the turnover here, however, is a reflection of the reversibility of the amino acid activation mechanisms of the cell rather than a demonstration of the utilization of the peptide in protein synthesis. With cell-free preparations from higher plants, Sissakian (141) has reported that glycyl peptides, labelled with C^{14}-glycine, are much more active than free C^{14}-amino acids in promoting incorporation of radioactivity into chloroplasts.

The investigations reviewed here are sufficient to indicate the possible importance of peptides in the synthesis of proteins. It is clear, however, that the ethanol-soluble peptides which have been studied in detail by Turba and others (165, 166) represent but a small part of the water-soluble peptide or proteose fraction of plant cells. As Folkes & Yemm (52) have shown, aqueous extracts of barley seedlings contain, in addition to the amino acids liberated by acid hydrolysis, large amounts of nitrogenous bases accounting for over 30 per cent of the total soluble N; the possibility cannot be excluded, therefore, that the so-called peptide fraction may include the amino acids from low molecular weight nucleoproteins such as have been found to be metabolically active by Webster (179) and by Hoagland *et al.* (69). More critical methods are clearly necessary for the separation and characterization of the peptide fraction of plants. Methods for the chromatog-

raphy of peptides have been reviewed recently by Moore & Stein (112). Feitelson & Partridge (47) have described a new ion-exchange reagent for peptide chromatography, and electrodiffusion processes for peptide separation have been developed by Synge (155).

Nucleic acids and protein synthesis.—The intricate relations between nucleic acids and protein synthesis have recently been reviewed in a symposium, edited by Crook (37), which dealt with the chemical and molecular structure of nucleic acid together with different aspects of protein synthesis in animal tissues and bacteria and in the multiplication of bacteriophage. Abundant evidence is assembled to show the close association of nucleic acids with protein synthesis, but remarkably little to indicate the nature and significance of this association in cell metabolism.

In yeasts and bacteria a close parallel between nucleic acid and protein synthesis in growing cells has often been demonstrated, but this does not necessarily imply a direct coupling of the synthetic mechanisms. The experiments of Pardee & Prestidge (121) and Yčas & Brawerman (185) have shown that synthesis of protein can be inhibited by agents such as chloramphenicol, while that of RNA continues and may under some circumstances be enhanced. A differential effect of inhibitors on the synthesis of proteins and nucleic acids in higher plants is indicated by the experiment of Kessler (79). It is probable that the interdependence of the synthesis of proteins and nucleic acids in growing cells results in part from the position of amino acids as precursors of protein and of the N-bases required for the synthesis of nucleic acids. The further studies by Levenberg *et al.* (87) of the biosynthesis of purines in animal tissues indicate that glutamine, glycine, and aspartic acid all contribute nitrogen to the ring structure. Other interrelations between the metabolism of amino acids and purines in bacteria have been investigated by Magasanik and others (100, 101).

Additional evidence of a direct relation between the synthesis of proteins and nucleic acids has been gained from work with intact tissues and cell-free preparations. Brachet (19) in experiments with young root tips of onion has shown that ribonuclease is a powerful inhibitor of both growth and the incorporation of labelled amino acids. Webster (176, 177) in a re-examination and extension of his earlier work has found further support for the view that the incorporation of amino acids into the microsome fraction of plant cells is dependent upon nucleic acid. In a brief report of recent experiments Webster (178) indicated that the effects of nucleosides in promoting amino acid incorporation was attributable to their conversion to nucleoside-5-phosphates which are RNA precursors. Moreover, mercaptopurines which inhibited amino acid incorporation appeared to do so by forming mercaptopuranoside-5-phosphate which was incorporated into RNA. These data, taken together with similar studies with microorganisms and animal tissues (56, 139) strongly suggest that the nucleoproteins of microsomes are important agents in the protein metabolism in the cell.

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Evidence of the interrelation of the synthesis of proteins and nucleic acid has been gained in studies of adaptive enzyme formation. The effects of exposure to ultraviolet light on the synthesis of nucleic acids and the formation of adaptive enzymes in yeast have been investigated by Halvorson & Jackson (61). It was shown that DNA synthesis was inhibited by short exposures, whereas longer exposure inhibited in a closely parallel manner adaptive enzyme formation, RNA synthesis, the incorporation of glycine-1-C^{14} and P^{32} into RNA, and the incorporation of amino acids into proteins. In nongrowing cultures of a mutant yeast Chantrenne (26) found that on exposure to oxygen, the induced formation of catalase, cytochrome *c*, and cytochrome peroxidase was associated with an increased incorporation of labelled adenine and uracil into RNA and DNA.

It has often been suggested that nucleic acids act as templates for specific assemblies of amino acids during protein formation. In this connexion DNA has generally been regarded as of chief importance owing to its pre-eminent rôle in bacterial transformations and in bacteriophage multiplication. However, Crick (36) has recently pointed out some of the difficulties in assigning a direct template action to DNA in the light of what is now known of its molecular structure. The possibility that RNA plays an equally if not more important part than DNA in protein formation is indicated by its association with microsomes which are very active in amino acid incorporaton *in vivo* and *in vitro*. Jeener (73) has briefly discussed this problem with particular regard to the multiplication of plant viruses.

The balance of evidence now available indicates that nucleoproteins, especially those of the microsomes, are relatively labile constituents of the cell which provide active centres for amino acid incorporation. It seems probable that these nucleoproteins may be stabilized to form structural proteins of the cell in a number of ways. In the formation of adaptive enzymes stabilization of specific protein structures may be brought about by enzyme-substrate relations, whereas the linking together of polypeptide chains by folding and cross bonding may lead to the more stable macromolecular units of reserve and structural proteins.

Cellular Organization in Relation to Nitrogen Metabolism

The importance of cellular structure in the regulation of metabolic processes is becoming generally recognized. The physical separation of some metabolic activities by barriers to diffusion and the close integration of others by virtue of the intimate arrangement of enzymes within the subcellular particles are but examples of structural factors which make it impossible to interpret the behaviour of cells merely from a knowledge of their total content of enzymes and substrates. It is for this reason that recent additions to our knowledge of the localization within the cell of the processes of nitrogen metabolism are briefly reviewed here.

The localization of enzymic processes involved in nitrogen metabolism.—

The problems concerned in the study of the localization of enzymes have been ably set out in the review by Goddard & Stafford (58). In critical studies attention must be paid to the possibility not only of the solubilization of enzymes by leaching from cell particles, but also of the adsorption, upon the particles, of enzymes that are normally soluble. Adequate attention has not usually been given to these possibilities in preparations from plant tissues; it is probable that some inconsistencies and discrepancies have arisen in this way.

Nitrite reductase has been reported by Medina & Nicholas (108) to be largely confined to a particulate fraction of *Neurospora,* while in higher plants nitrate reductase has been shown by Evans & Nason (45) to be present in chloroplasts where its function may be linked with photoreduction. Davies (39) in a study of mitrochondria from peas has demonstrated the presence of glutamic dehydrogenase as well as most of the enzymes involved in the Krebs Cycle, while in particulate preparations (mainly mitochondria) from cabbage, Freebairn & Remmert (53) have found glutamic-oxalacetic transaminase. A probable glutamic acid-proline interconversion is recorded for barley mitochondria by Folkes & Yemm (52). It therefore seems likely that as in animal cells the processes of amino acid synthesis and interconversion, together with the synthesis of the keto acid substrates of these processes, occur within the mitochondria. Some doubt, however, has been cast upon the location of glutamine synthetase; in contrast to the views of Webster (see 175), Loomis (90) maintained that this enzyme was not located in the mitochondria but was soluble as was also glutamyl transferase. Critical reexamination of this point appears to be necessary. The extent to which chloroplasts reduplicate some of the biochemical functions of mitochondria is also in question. In a short account of the work of his school on the biochemical properties of plastids, Sissakian (141) reported that cyclophorase activity could be demonstrated in isolated chloroplasts. Phosphorylation in chloroplasts has been reviewed recently by Arnon (6); here it appears that the mechanism is similar to, but far from identical with, the mitochondrial pathway for oxidative phosphorylation.

The problem of protein synthesis and the evidence for the microsomes as the chief site of that synthesis have been discussed earlier in this review. It is worth noting, however, that other organelles may also synthesize protein. Allfrey *et al.* (2) have shown that amino acid incorporation and the synthesis of nucleoprotein can occur in isolated nuclei from mammalian thymus tissue, and the role of the nucleus in the control of protein synthesis has been considered by Brachet (18). Sissakian (141) states that when ATP is supplied to isolated chloroplasts, protein is synthesized at the expense of the nonprotein nitrogen, chiefly peptides; he was able to show incorporation of C^{14} from simple di- and tri-peptides. It is clearly reasonable to suppose that, although the majority of protein or polypeptide synthesis is probably localized in the microsome, other self-replicating organelles of the cell

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have some protein synthesizing or protein organizing properties. Such ideas would explain the very even distribution of N^{15} among the proteins of the plastids, mitochondria, microsomes, and supernatant which was observed by Cocking (31) in young barley seedlings after a few hours' growth in labelled ammonium phosphate solution.

Metabolic evidence for the spatial separation of amino acid metabolism and protein synthesis.—In a study of the kinetics of ammonia utilization by *Azotobacter,* Burma & Burris (21) transferred cells which had been grown exponentially on atmospheric nitrogen to a solution containing $N^{15}H_4^+$. The free amino acids of the cells became labelled within the first minute, but there was a slight lag before the amino acids of the proteins became labelled also. From this it was deduced that protein synthesis in the sense of amino acid incorporation was physically remote from the sites of amino acid synthesis. A further demonstration of the separation of cellular processes has been the analysis by Steward *et al.* (148) of their complex data on amino acid and carbohydrate metabolism in carrot tissue supplied with C^{14}-labelled substrates. For any particular metabolite Steward *et al.* attempted to calculate the specific activity of the carbon which was in transit to and from the metabolite in question and from the results were able to demonstrate that in some cases two distinct pools of the metabolite must exist within the cell. It was clear that many of the amino acids of the cell were not in equilibrium with the sites of protein synthesis and that there was closer metabolic contact of protein synthesis with the carbohydrates of the cell than with the total pool of free amino acids. The general conclusions to be drawn from these results will be discussed later, and it is only proposed to point out here the importance of the cell vacuole in the accumulation of amino acids and peptides by storage organs such as the carrot. Such mature tissues have relatively high levels of free amino acids and peptides when compared with rapidly growing tissues such as in young seedlings. The stored amino acids may not be in equilibrium with the true metabolic pool of the organism and so may take little part in protein synthesis under conditions of plentiful nitrogen supply; the amino acids for protein synthesis are instead synthesized *de novo* from inorganic nitrogen and carbohydrate. Some evidence for the spatial separation of newly synthesized and stored amino acids in roots cames from the work of Frenzel (54). He found that sterile water cultures of sunflowers lost appreciable quantities of amino acids into the culture solution but only when supplied with inorganic nitrogen; roots in nitrogen-free cultures lost no organic nitrogen although their tissues were quite rich in stored amino acids.

Interrelations of Nitrogen Metabolism with Carbohydrate Metabolism and Respiration

There is still much controversy as to the general ways in which metabolism is regulated in growing plant cells. Two distinct approaches have already been noted. On the one hand, a continuous protein cycle is visualized

as a mechanism which regulates the breakdown of carbohydrate and respiration; it is chiefly from this standpoint that the alternative hypothesis of protein synthesis has been developed (149). On the other hand, the regulation of carbohydrate breakdown and cell respiration has often been attributed to their coupling by means of phosphorylation and oxidation-reduction to endergonic processes of growth. This concept of the regulatory mechanisms has been recently discussed in its general aspects by Lardy (86) and, with particular regard to the synthesis of amino acids and proteins, by Yemm & Folkes (52, 188). In this section evidence bearing on the two hypotheses will be examined.

The dynamic state of cell proteins and the protein cycle.—The early experiments of Vickery *et al.* (170) with isotopic nitrogen indicated the dynamic state of cell proteins in plants and were interpreted as evidence of a protein cycle. There are, however, several difficulties in the interpretation of these and other similar data. In growing plants and their organs it is probable that a steady state is rarely established; the breakdown of proteins generally predominates in senescent organs, the products being utilized for protein synthesis in young and meristematic parts of the plant. In this connexion, Dorner *et al.* (44) have followed the fate of two major protein fractions separated by electrophoresis from tobacco leaves at different stages of growth. They found that the fractions were continuously changing so that at no time was there a steady state in which synthesis was balanced by catabolism. Apart from a continuous movement between different parts of the plant, it is by no means certain that the incorporation of isotopically labelled amino acids into proteins necessarily involves complete breakdown and resynthesis of proteins with extensive oxidation to carbon dioxide of the carbon skeletons of amino acids; it is, however, well established that such oxidations occur in starving or senescent organs of plants (151). The rate of incorporation of N^{15} into the proteins of excised barley roots investigated by Yemm & Willis (189) was relatively constant over a period in which wide fluctuations of respiratory rate took place. It is, therefore, unlikely that in this tissue the high rate of respiration observed during the assimilation of nitrogen was due to an increase in the rate of a protein cycle. As already noted on p. 260, exchange reactions between amino acids and labile proteins could readily account for the incorporation of N^{15} into the protein under experimental conditions such as those outlined above.

In a search for more critical evidence of the operation of a protein cycle, unicellular organisms offer better experimental material than tissues of higher plants. From extensive experiments with *E. coli* Roberts *et al.* (133) concluded that there was no evidence of a protein cycle in the growing cells. It may be noted here that although Roberts *et al.* could account for only part of the CO_2 produced by *E. coli* as derived from the tricarboxylic acid cycle this is not, as was suggested by Steward & Pollard (149), evidence of the formation of CO_2 by a protein cycle; Roberts *et al.* were in fact able to show convincingly that the extra CO_2 could be attributed to a pentose phosphate

pathway. In other experiments with *E. coli* Mandelstam (102) also found no evidence of protein turnover during exponential growth, but he considered that this might be due to lack of exchange between the amino acid pool and the external medium under these conditions. By using a leucine-requiring mutant in media free from either leucine or ammonia, he was able to measure C^{14}-glycine incorporation or leucine release in nongrowing cells. The results indicated that up to five per cent of the protein might turn over per hour, but Mandelstam was unable to decide whether this represented a real difference between the metabolism of growing and nongrowing cells. In any event exchange reactions might account for the turnover, as indicated by Gale (56) in his studies with other nongrowing bacteria. This interpretation is supported by Mandelstam's recovery of C^{14}-leucine from the medium after addition of normal leucine to a culture containing labelled leucine in the cell proteins. Such an observation is clearly inconsistent with the operation of a protein cycle; no direct evidence in support of a cycle has so far been obtained with growing cells of bacteria.

In an interpretation of experiments with tissue cultures of carrot, Steward & Bidwell (147) have emphasized several features which support the alternative hypothesis of protein synthesis operating with a protein cycle. The fact that C^{14}-glucose labelled the amino acids of the cell proteins more effectively than C^{14}-glutamine was regarded as evidence that carbohydrate was the chief source of carbon for protein synthesis by a direct pathway not involving amino acids. Further support for this view was drawn from the observation that a wide variation in the specific radioactivity of different amino acids in the tissues was not reflected in the labelling of the amino acids of the proteins. Some of the difficulties in the interpretation of these data arise because of the complexity of cell organization and have already been noted on p. 268. In carrot tissue cultures, as in storage tissues of plants, relatively large amounts of free amino acids and amides may accumulate as reserves in the vacuolated cells and may not be readily accessible to the centres of protein synthesis. Thus, if exogenous supplies of glutamine are stored as reserves in the cell, glucose may provide a more directly available source of carbon for protein synthesis. Similarly, the increases of reserve amino acids by synthesis or interconversion would lead to differences between the labelling of the free amino acids and of those combined in the tissue protein. Decisive evidence of a protein cycle is even more difficult to obtain from the data on the labelling of CO_2 when C^{14}-glucose, C^{14}-glutamine, or C^{14}-γ-aminobutyric acid was supplied to the tissue cultures. The much greater effectiveness of C^{14}-glucose in labelling the CO_2 clearly indicates that a large part of the respiratory substrate is derived from glycolysis. This is particularly evident in the rapidly growing cultures in which there is a close relation between the labelling of the sugars in the tissues and that of the CO_2. The relatively lower labelling of CO_2 when C^{14}-glutamine or C^{14}-γ-aminobutyric acid was supplied is consistent with the view

that these metabolites are less accessible to the centres of cell respiration and of protein synthesis. In view of other strong evidence that amino acids and amides do not freely equilibrate in different parts of organized cells (see p. 268), it seems clear that the data do not provide unequivocal evidence of the operation of a protein cycle. In further experiments with carrot tissue Steward & Pollard (150) have recently reported that proline can be directly utilized in protein synthesis. When C^{14}-proline was supplied to growing cultures, almost all of the radioactivity was found in the proline and hydroxyproline residues of the tissue protein, very little C^{14} being lost as CO_2. The striking difference between glutamine and proline in this respect may be partly due to their different positions as precursor and end product in the glutamic acid family of amino acids.

The regulation of carbohydrate metabolism and nitrogen assimilation.— One of the most direct demonstrations of the close connexion between nitrogen metabolism and cell respiration is the great increase in respiratory activity when nitrogen is assimilated. As already indicated on p. 250, very active assimilation occurs in cells and tissues starved of nitrogen but rich in carbohydrates. Under these conditions a rapid synthesis of amino acids and amides is accompanied by a rise of the respiration rate which in some organisms increases two to three times within a few minutes of supplying the nitrogen; data of this kind have been obtained with excised roots (181), *Chlorella* (156), and *Torulopsis* (188). In all these cases an extensive breakdown of carbohydrates was demonstrated, usually in amounts adequate to account for both the CO_2 and the carbon skeletons of the amino acids and other products of nitrogen assimilation. As a result of the importance of carbohydrate breakdown, attempts to explain the phenomena have centered upon mechanisms of the regulation of glycolysis. Syrett (156) suggested that the utilization of ATP for the synthesis of glutamine was of chief importance, whereas Yemm & Folkes (188) offered a more comprehensive interpretation which included not only the effects of phosphate and phosphate acceptors, but also oxidation-reduction changes associated with reductive amination, and the diversion of carbon skeletons for the synthesis of amino acids. These closely coupled mechanisms appear to account satisfactorily for the regulation of glycolysis and cell respiration under a wide range of physiological conditions, including the assimilation of nitrogen.

Other ways in which oxidation-reduction changes associated with nitrogen assimilation may regulate the metabolism of carbohydrates have been indicated by Bidwell *et al.* (10). They found that C^{14}-glucose supplied to wheat leaves did not label pyruvate and its derivatives when the leaves were kept in the light although in the dark these compounds were strongly labelled. The blockage was attributed to the effect of triosephosphate dehydrogenase activity of the strongly reducing cell environment during photosynthesis. When ammonium nitrate was supplied in the light, the blockage

was removed, suggesting that a diversion of reducing power to amino acid synthesis allowed the oxidation of triosephosphate to proceed. It is also possible that utilization of glycolysis products to provide carbon skeletons for the synthesis of amino acids is involved here.

The origin of the carbon skeletons of amino acids in glycolysis or in photosynthesis has been extensively studied with C^{14}-labelled substrates. Strong evidence of the importance of the tricarboxylic acid cycle in this connexion was obtained in the experiments of Roberts *et al.* (133), Cowie & Walton (35), and Vavra & Johnson (169) with heterotrophic microorganisms. The relation between the formation of amino acids and photosynthesis in leaves of higher plants was explored by Bidwell *et al.* (10) and by Nelson & Krotkov (116). There are several indications that special mechanisms operate to replenish C_4-acids when they are extensively used for synthesis of amino acids. In experiments with *Chlorella* Syrett (159) found that carbon dioxide fixation (presumably $C_3 + C_1 \rightarrow C_4$) was increased during nitrogen assimilation; similar effects were obtained by Strauss (152) using succinate requiring mutants of *Neurospora*. Under anaerobic conditions $C_3 + C_1$ reactions appeared to be especially important for the synthesis of amino acids in the experiments of Vavra & Johnson (169) with baker's yeast. On the other hand, Nelson & Krotkov (116) concluded that $C_2 + C_2$ condensation operated in aspartic acid synthesis when bean leaves were supplied with ammonium nitrate during photosynthesis. Kornberg (82) from his work with *Pseudomonas* grown on acetate as the carbon source suggested that only about 15 to 17 per cent of the carbon of the protein was derived from CO_2 fixation, the rest being obtained by way of the glyoxylate cycle. The operation of this cycle has recently been demonstrated in higher plants by Kornberg & Beevers (83) so that it is possible that here, too, CO_2 fixation may be less important in nitrogen assimilation than was previously thought. Another metabolic rôle of glyoxylate is suggested by the work of Rabson & Tolbert (128) with excised leaves; glyoxylic acid, presumably derived from pentose, was used in preference to photosynthetic phosphoglyceric acid for the synthesis of glycine and serine. Thus there are indications that the pentose pathway of carbohydrate breakdown, as well as glycolysis, may be closely coupled to the synthesis of some amino acids.

Protein synthesis and the regulation of cell metabolism.—It is clear that in addition to the relationships just discussed much more complex interactions between carbohydrate and protein metabolism may occur in growing cells in which interconversion of amino acids and new synthesis of proteins are taking place. Although reduplication of cell proteins is a basic feature of cell growth and differentiation, it is only one of a number of anabolic processes by which organic and inorganic materials are assimilated. All these processes are in part dependent on carbohydrate metabolism and

cell respiration; they may react upon regulatory mechanisms which control glycolysis.

The relative merits of hypotheses based upon the protein cycle or upon the regulation of glycolysis have been discussed by Folkes & Yemm (52) in a study of respiration and protein metabolism in seedlings of barley. Here, extensive interconversions of amino acids accompany the synthesis of new proteins in the embryo at the expense of storage proteins of the endosperm. In some respects the data on CO_2 production of the seedlings are consistent with a protein cycle in which amino acids supplied by the endosperm are oxidized to CO_2 and the ammonia, thus released, utilized with carbohydrate for synthesis of protoplasmic proteins in the embryo. However, other interpretations are possible. Experiments with grain of different nitrogen contents showed that the carbon dioxide output per unit of embryo protein-N was independent of the nitrogen content of the seedlings and followed an identical course in all experiments, increasing at first and later declining to a steady value. As protein-N of the embryo may be regarded as a comparative measure of the enzyme complement of the growing tissue of the seedling, the constancy, at any particular stage of germination, of respiration per unit of protein may result from a particular level of metabolic activity and depend upon the energy requirements of assimilation and synthesis within the embryo. These latter requirements are likely to be greatest during the rapid growth which occurs in the early stages of germination; it is consistently found when compared on a protein basis that the most intense respiratory activity coincides with the highest rates of protein synthesis and assimilation of new materials in the embryo. Some support for the concept of regulatory mechanisms linking protein metabolism and respiration in barley seedlings can be gained from the data of Bjørnseth *et al.* (12) who investigated the germination of grain subjected to various levels of irradiation with thermal neutrons. The irradiated seeds were apparently depleted in DNA and this led to reduced growth, protein synthesis, and respiration as compared with the controls. However, as in the experiments of Folkes & Yemm (52) the rate of respiration was closely related to the protein content of the seedlings despite marked differences in other nitrogenous constituents.

Recent investigations of protein synthesis in microorganisms have indicated the complexity of the changes of cell proteins in relation to growth. For example, Sims (140) in a reexamination of the influence of carbohydrate and nitrogen supply on growth in *Torulopsis* has shown that cells with plentiful reserves of endogenous carbohydrate did not divide and grow when supplied with ammonium phosphate in spite of high rates of respiration and of nitrogen assimilation. The increase of protein-N in the yeast, earlier reported by Yemm & Folkes (188) under similar conditions, was due to a nucleoprotein or polypeptide fraction which could be extracted

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from the cells, together with much of the RNA, by citrate buffers; no increase could be detected of structural proteins insoluble in citrate. Synthesis of these latter proteins occurred only when exogenous sugar was supplied and was accompanied by marked changes in the free amino acids in the cells including increases of aspartic acid, alanine, and basic amino acids. Sims has further shown that the amount of citrate-soluble protein fluctuates during different stages of growth; there is a marked decline as cells pass out of the exponential phase and a restoration of the level during the lag phase preceding cell division. An accumulation of nucleoproteins was found by Hotchkiss (72) in *Staphylococci* supplied with glucose and an incomplete mixture of amino acids on which the culture did not grow. In *E. coli* Dagley & Sykes (38) detected by ultracentrifugation a distinctive RNA-protein fraction which changed in amount under different physiological conditions. In starved cells the fraction disappeared and was restored in a complete medium which supported growth; it persisted when only glucose was exhausted from the medium. These investigations indicate the importance of concomitant syntheses and particularly those of nucleic acids which appear to be closely related to protein synthesis and cell growth.

Several other studies in addition to that of Sims (140) have emphasized the decisive effects of carbohydrates on protein synthesis and growth. Kandler & Ernst (75) found that organic acids could not replace glucose as a carbon source for sustained assimilation of nitrogen by *Chlorella*. Experiments by MacMillan (98) with the fungus *Scopulariopsis brevicaulis* showed that it was unable to synthesize protein unless glucose was supplied in the medium. In this and some of the other studies with microorganisms, there are indications that external supplies of sugar are necessary for protein synthesis even when a plentiful supply of endogenous carbohydrate is present.

In addition to the data discussed by Steward & Bidwell (147), the possibility that auxin and other growth substances regulate protein metabolism has been further examined. Reinhold & Powell (131) investigated the action of indoleacetic acid on the uptake of C^{14}-glutamic acid and C^{14}-glycine by segments of sunflower hypocotyls. They found that the auxin increased the incorporation of C^{14} in the segments, resulting in a greater specific activity of the total carbon. Higher rates of respiration were also induced and the CO_2 had a greater specific activity. These observations could be interpreted in terms of the effects of auxin on cell permeability, but a similar explanation does not hold for work with another growth substance by Akers & Fang (1). They examined the effect of 2,4-D on the incorporation of C^{14} into the total glutamic and aspartic acids of kidney bean plants supplied with $C^{14}O_2$ for photosynthesis. Treatment with 2,4-D caused a fall in protein level in the plants, but a large increase in labelling of the dicarboxylic acids as compared with the controls. Akers & Fang (1) interpret their results as indicating an increased turnover of protein in the presence of

2,4-D; greater meristematic activity coupled with breakdown of proteins in older tissues might well account for these results.

Conclusions

At present conclusive evidence is lacking at many critical points in the metabolism of amino acids and proteins in plants; the rival claims of the protein cycle as against regulatory mechanisms of glycolysis cannot as yet be decided with certainty. But a basic similarity in the pattern of metabolism in plants and other organisms continues to be revealed. The value of comparative methods in biochemistry, firmly established in studies of glycolysis and cell respiration, extends to many aspects of nitrogen metabolism. Against this background the amino acid hypothesis and the concept of regulation of glycolysis and cell respiration by their coupling with endergonic processes seem to offer the most comprehensive interpretation of the relation between carbohydrate and nitrogen metabolism and the most useful guide for further studies.

It is tempting to suggest that distinctive features of protein metabolism in plants do not depend so much on basic differences in the biochemical mechanisms of plants as upon special features of their cell organization. Storage of amino acids and amides, probably in the cell vacuole, often plays an important part in the economy of nitrogen in plants. It is perhaps significant that the alternative hypothesis of protein synthesis has been mainly developed from experimental work with storage tissues where dual functions may obscure the relations between amino acids and protein synthesis. The recognition and investigation of the complexities of cell organization in plants are likely to be of increasing importance in the study of their nitrogen metabolism.

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AUXIN USES IN THE CONTROL OF FLOWERING AND FRUITING[1,2]

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While the practical aspects of auxin usage have been reviewed in books by Audus (6), Tukey (166), and Leopold (93), this is an area of specialization and rapid change where the written word quickly becomes modified in practice. In the present review we will discuss auxin uses to alter flowering and fruiting processes, and an attempt will be made to relate the practical applications to the physiological science. In order to minimize repetition, emphasis will be laid upon the developments since the most recent review of the subject (93).

FLOWERING

Auxins have proven to be useful or have shown promise of becoming useful in the modification of flowering through four types of action: (*a*) the alteration of earliness with the application of the compounds to seeds or seedlings; (*b*) the induction or promotion of flowering; (*c*) the delay of flowering time; and (*d*) the influencing of flowering through indirect morphological expressions.

Altering earliness.—After the original observations that treatments of seed with auxins could increase earliness (27, 165), only scattered reports were made of the successful use of auxins to this end (26, 93). Kruyt (89) has actually counted the number of reports published between 1936 and 1951 which did or did not show increased earliness following the treatment of seeds with auxins, and in 121 reports increases in earliness were of approximately equal frequency with lack of increases. Some promise of greater dependability for this auxin use appeared in 1953 when Leopold & Guernsey found that treatment of seeds of various species of crop plants with auxin followed by low temperature——typically 4°C. for 4 to 15 days ——produced quantitative increases in earliness of soybean, oats, corn, barley, and peas (95 to 98). Even when combined with the cold treatment, however, the application of auxin for increased earliness has not proven to be consistent as field tests by Leopold's group (92) have shown. The same pattern of gains and no gains in earliness seems to hold for reports of trials attempting the temperature control following auxin treatment. While Asana *et al.* (3) report that wheat responds to auxin treatment of the seed with increased earliness and vigor, Chakravarti (24) found only inhibitory effects of several auxins upon vernalization of flax. Later ex-

[1] The survey of literature pertaining to this review was concluded in October, 1957.

[2] The following abbreviations are used: 2,4-D for 2,4-dichlorophenoxyacetic acid; chloro-IPC for chloroisopropyl-*n*-phenylcarbamate.

periments by Chakravarti *et al.* (26) produced large gains in earliness and yields of mustard when auxin was applied just before the chilling treatment, and no gains when applied after the chilling. Moore (117) reported a similar situation in cabbage. He found that treatment of cabbage plants with a weak auxin (*p*-chlorophenoxypropionic acid) before chilling produced large increases in earliness and flowering while similar auxin treatment during or after chilling time were without effect or were inhibitory. A slightly different response pattern has been reported for Brussels sprouts which respond to dilute auxin applications applied during the cold vernalization period by earlier flowering and apparent shortening of the juvenile phase; similar auxin treatments before or after the cold treatment were not significantly effective in increasing the earliness of flowering (186).

Some reservations should be kept in mind as to whether the reported influences of auxin upon earliness are a consequence of auxin action, or whether they are a consequence of secondary actions of the applied chemicals. It is pertinent to note that thiamine and triiodobenzoic acid have the same effects on the earliness of peas as do the auxins tested (98), that maleic hydrazide can produce early flowering in cabbage when applied during cold treatment (117), and that N-tolylphthalamic acid can increase the earliness in tomatoes in a manner apparently parallel to the increase in earliness by low temperatures (101, 164, 182, 183). None of these four substances are auxins (93).

Induction or promotion of flowering.—Of course the classic case of the induction of flowering with auxin is the pineapple. Almost simultaneous reports from Hawaii and from Florida (30, 33) first described the remarkably complete response of this species to auxin. At present nearly every field of pineapple in Hawaii is treated with sodium naphthaleneacetate at some time during the growing cycle. Typically the chemical forcing of flowering is carried out on the second or ratoon crop in order to reduce the inherent variability in time of harvest of this crop. In the Caribbean area, 2,4-D is more generally utilized (132). The development of this use of auxin is reviewed elsewhere along with a description of some of the problems of fruit culture associated with the use of the auxin treatment (93).

Another fruit crop which is yielding to auxin control of flowering is the litchee. This subtropical tree apparently does not establish flower primordia during periods of active vegetative growth, and in Hawaii the time of probable floral initiation is generally interrupted by vigorous vegetative growth (125). Consequently, flowering is sporadic or in some locations quite lacking. Shigeura (145) observed that auxin sprays were able to cause flowering in this species, and later Nakata (125) studied the auxin response in greater detail. He established that naphthaleneacetic acid, applied in wheat flour to avoid toxic effects of the usual auxin carriers, retarded the vegetative growth flushes and resulted in flowering. He also found that girdling, low temperature treatment and short photoperiods were each able to bring

about flowering as well. One is led to wonder if each of these treatments induced flowering as a consequence of retardation of vegetative growth.

Another use of auxin in the control of flowering, helpful to the plant breeder, is the control of flowering in sweet potato with 2,4-D. In this case (83, 84) surprisingly large amounts of auxin are applied so that severe injuries are obtained on the foliage, and flower initiation follows. The 2,4-D treatment was found to be effective on each of the varieties tried including the desirable and persistently nonflowering Jersey types. The only other known means of bringing the Jersey sweet potatoes into flower is to graft them onto flowering stocks of other varieties from which the flowers and fruits are removed (91).

While flower initiation has been induced with auxin in some other species, this has been done only under specialized conditions approaching those of photoperiodic induction (102) and is of physiological interest rather than of agricultural usefulness at present.

Mention should also be made here of the uses of gibberellins for the induction of flowering. These compounds are not auxins, but they offer great promise for the effective control of flowering in a rather large number of crop plants (158).

Preventing or delaying flowering.—While most of the promotive effects of auxins on flowering are subtle and elusive to experimentation, the inhibitory effects are obvious and strong. Judging from the frequent reports of auxin inhibition of flowering, it would seem that most or perhaps all plants can be so inhibited.

Superimposed on the flowering inhibition, however, are the formative or rather deformative effects of auxins. Because of the toxic responses of plants to auxin treatments at concentrations which inhibit flowering, auxin has found no real usefulness in the prevention or delaying of flowering. Whereas high concentrations of auxin can effectively prevent the flowering of pineapple (30), attempts to utilize this action on plantations have always severely damaged the crop. While there is considerable evidence that the natural flowering of many species of plants is inhibited by high endogenous auxin concentrations [e.g., von Denffer (48)], attempts to reach this inhibitory state with applied auxins have uniformly failed in that the plants are damaged before effective control is obtained [e.g., Rice (137)].

One exception to this may be the prevention of bolting of celery and cabbage by the application of *p*-chlorophenoxypropionic acid during cold periods which would normally induce the flowering state. Such sprays apparently do not cause noticeable formative effects on celery seedlings (179, 181), but the control of flowering is also quite precarious in that slight differences in the time of application will shift the effect from a delaying to a promoting action (31). Similar effects are obtainable with *o*-chlorophenoxypropionic acid on cabbage (117).

Attempts have been made to forestall the opening of fruit buds in spring

to protect them from early frosts. These have been essentially unsuccessful (93).

By using inhibitors other than auxins, some success has been achieved in preventing flowering. Maleic hydrazide was recognized as an inhibitor of flowering in 1950 (126) and since that time it has been found to control bolting in onions. This is useful as bolting spoils the quality of the onion bulb (28). In the Indian radish, the huge roots which may weigh as much as 10 lb. each, become bitter and woody after flowering; in this case, also, the application of maleic hydrazide has provided an effective control of bolting and flowering (29). What distinction there might be between the species which are prevented from flowering by maleic hydrazide and those which are promoted to flower is paradoxical.

Altering flowering by morphological modification.—In addition to the cases described above, several types of applications of auxins and other growth regulators have been devised for use in increasing the numbers or the quality of flowers. For example, flowering of tomato has been greatly promoted by triiodobenzoic acid (168) and by N-tolylphthalamic acid (164) through mechanisms that have been interpreted as the prevention of correlative inhibition of flowering (185). Somewhat similar floral promotions have been obtained with beans using the benzoic acids (64) or 2,4-D (173) and with lima beans using 2,4-D (112). The possible mechanism of these promotive effects will be discussed in the next section, but for the purpose of the present discussion they may be considered together as floral promotion through a disruption of morphological development rather than through more direct effects on the flowering stimulus.

Many reports have indicated that triiodobenzoic acid effectively promotes flowering in tomato (63, 168, 189) but in spite of a quite dramatic ability to increase flowering, its application has not achieved practical use. This is probably the result of the distortion effects suffered by the tomato plants and suppression of leaf and stem growth when sufficient triiodobenzoic acid is applied for the flowering response to be altered. The subsequent productivity of the plant is impaired.

The promotion of flowering in tomato with N-tolylphthalamic acid is actually in use for the production of early crops of greenhouse tomatoes and less frequently for later tomatoes (183). Distortions to the plant are usually limited to a flagging of the leaves for a few days followed by darkening of the foliage. Death of the growing point may also occur, but new laterals soon take over. The chemical treatment of tomato plants can double or triple the numbers of flowers in the cluster differentiating at the time of application.

Another type of chemical modification of growth which can benefit flowering is the use of growth regulators to break apical dominance. Many floricultural crops require the removal of growing points in order to force out desirable lateral buds. This process, called pinching, is used most com-

monly in commercial production with chrysanthemums and carnations. Maleic hydrazide has shown some promise as a substitute for pinching in both crops (13, 14). Variable responses are sometimes obtained with this chemical resulting in toxic effects to leaves and flowers (113), undoubtedly due to variations in penetration of the chemical into the foliage. When more uniform penetration is obtained (190), this use of maleic hydrazide as a growth regulator may be very much enhanced. The chemical may also be useful for forcing lateral buds of roses (4) and peas (116).

As described in earlier reviews (6, 93) auxins and other growth substances can effectively alter the sex of flowers. Male sterility can be produced (127, 135) or the number of male flowers can be reduced (180) by using maleic hydrazide. Auxins can increase the proportion of female flowers especially in the cucurbits (60, 90, 129). It is notable that either low temperatures or auxin may bring about the same type of modification of sex ratios (60, 129, 136). The temperature effect has been pursued further to determine whether cold could increase the auxin promotion of female flowers after seed treatment in the manner of the combined auxin cold treatment of seeds. These experiments established that chilling the germinating seeds of cucumber lowered the number of the node that produced the first pistillate flower, and pretreatment of the seeds with naphthaleneacetic acid considerably enhanced this response (60).

On the basis of flowering effects.—At the present time there is no clear evidence that auxins constitute a part of the flowering stimulus itself. There is some evidence that growth substances may inhibit the translocation of the hypothetical flowering stimulus and some less direct evidence that they may contribute to the destruction of the flowering stimulus [Salisbury (142)]. In addition, it is reasonable to assume that inhibition of flowering by auxins may be a consequence of non-specific inhibitions or toxicities.

Some promotion of flowering by auxins may result from the influence of auxin upon vegetative growth. This speculation may be supported by examples from each of the types of auxin promotive effects discussed above. It may be recalled, for example, that Cholodny (27) suggested that the stimulus of vernalization was a consequence of the suspension of vegetative growth of seedlings during chilling treatment. Again it is known that flower induction in the pineapple is associated with an interruption in the growth of the young expanding leaves [see Leopold (93)]. Auxin treatments to induce flowering produce a similar growth break. Nakata (125) has described inhibition of flowering in litchee by vegetative growth flushes, and the prevention of the flushes with auxin treatment may be the means of floral promotion in that species. Fisher & Loomis (59) found that in photoperiod experiments with soybeans floral promotion could be obtained with nicotine sulfate or with triiodobenzoic acid sprays, and this floral promotion was associated with growth inhibition. In fact, the simple operation of repeated removal of the apical buds or the young expanding leaves had

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the same promotive effect. These experiments closely parallel the procedures and results of de Zeeuw (185) who more than doubled the flower initials in tomato by the repeated removal of expanding leaves. He also discovered that the promotions of flowering by triiodobenzoic acid and N-tolylphthalamic acid were obtained only if these expanding leaves were left on the plant suggesting that promotions by these chemicals were perhaps alleviations of floral inhibitions invoked by the growth of the leaves.

The improvement of flowering in lima bean (112), bean, (65, 173) and chrysanthemum (167) by chemical treatments which arrest or even kill the apical meristems can be interpreted as fitting well with the suggestion that the chemicals are acting through a retardation of vegetative growth.

An earlier suggestion has been made that applied auxins and other growth regulators may increase or induce flowering through a lowering of the functional level of auxin in the plant meristems. This suggestion was first made by Bonner & Bandurski (17) and has been supported by Gowing (66) with experiments on flower induction in pineapple using naphthaleneacetic acid. The assumption is that auxins act in opposition to flowering, and promotions are obtained with auxin applications because the applied chemicals have activated the auxin-destroying mechanisms in the plant leading to a lowered auxin level; or alternatively, the applied auxin competes with the more powerful endogenous auxin and lowers its effectiveness. First of all, there is a quantity of evidence to suggest that treatments which lower the auxin content of plants can, in fact, promote flowering. For example, decapitation (134, 167), treatment with ultraviolet light (49), or x-rays (99) are all devices which should lower the auxin content and which do promote flowering under some circumstances. Eosin is known to bring about auxin oxidation by light, and its application to plants can increase flowering (103). On the other hand, some reservations appear to be in order for applying this interpretation to experiments in which applied auxins have stimulated flowering. Analyses of the free auxin contents of plants, seeds or seedlings after auxin application show either increases or no change in the amount of free auxins in the tissues, rather than decreases (7, 18, 76, 77). Until experimental evidence for such a decrease in effective auxin content is available, the suggestion must be very tentative.

One other physiological means through which auxin applications may bring about floral promotion is through the promotion of bud growth. It is well known that there are auxin levels which will promote bud growth (see 93), and if the promoted buds are reproductive, it is logical that there should be some acceleration of the date of flowering or of the development of flower buds. Experiments describing such a promotion have been published by Hussey & Gregory (85) for barley and by Sircar & Kundu (148) for rice. Salisbury (142) has identified a promotive effect of auxin on flowering in cocklebur as being identical with the promotive effects invoked by the presence of growing buds.

One more physiological comment should be added here: the question might be entertained as to whether the effects of chemicals such as the phenoxyacetic acids and related materials on flowering are due to their auxin properties. One should bear in mind that the promotion of earliness described in the first section can be had with triiodobenzoic acid and thiourea; that the forcing of flowering in pineapple can be had with ethylene and acetylene (100, 140); or even with β-hydroxyethylhydrazine [see (66)]. The synthetic auxins including indoleacetic acid are very diverse in their biological activities. The assumption that the effects of one of these chemicals on flowering are due to its auxin activity is no more reliable than the postulation that its effects on metabolism, on photosynthesis, or on ripening of bananas are necessarily a consequence of its auxin properties.

Fruit Set

The first crop to achieve wide usage of auxins for fruit set was the tomato. In this plant the application of auxin can effectively replace the need for pollination of the flower; indeed, the commercial use of auxins for setting these fruits is tailored specifically to improve set under such conditions as are unfavorable for set with pollen. The consequent development of seedlessness has no merit in itself.

Pollination in tomatoes fails under three main conditions: (*a*) pollen is made inviable by low temperatures or by weak light conditions; (*b*) pollen fails to reach the stigma under greenhouse conditions in the winter when light intensities are low for prolonged periods, when the nitrogen supply to the plants is excessive, or when temperatures are high; these conditions, leading to the development of excessively long styles, each appear to produce a condition of excessively high levels of available nitrogen in the plant; (*c*) tomato seedlings with a small number of mature leaves fail to respond to pollen even when it successfully reaches the stigma. These three conditions leading to failure of pollination are each alleviated by auxin applications to the flower clusters [see review (93)].

High temperatures such as are experienced during the summers in the central and southern parts of the United States bring about a different failure of tomato fruit set which is a major limitation to the productivity of the crop in many areas. Unfortunately the application of auxin under these conditions fails entirely to improve set (86, 118). It has been reported that shading the plants can restore the responsiveness of the flowers to auxin applications (118).

Among other herbaceous species many members of the Solanaceae and Cucurbitaceae will set fruit in response to an auxin spray.

Among tree fruits, auxins hold great promise for usefulness in setting fruits of Calimyrna fig (40). The auxin replaces the need for male trees in the orchards as well as for the culture of the caprifying wasp which is

essential for the successful pollination of the flowers. At present the use of auxin sprays is not widespread, for the most effective auxin (*p*-chlorophenoxyacetic acid) produces a seedless fruit which is not considered desirable, and the auxin which produces seed-like structures in the fruits (benzothiazole-2-oxyacetic acid) (34a) is not as effective in fruit set.

Auxins have not achieved usefulness in the setting of other tree fruits. It has been pointed out (101a, 128) that parthenocarpic set is most effective with fruits containing large numbers of seeds, though there are exceptions to this generalization. Fig is of course outstanding in this quality, and most varieties of figs set quite entirely without pollen. The same is true of the banana, most varieties of which are parthenocarpic; and those which are not can be set with auxin (146). Fruits with somewhat fewer seeds, as for example the pear and the apple, only rarely set fruit parthenocarpically and only rarely respond to auxin treatment with improved fruit set. In this connection it is curious to note that parthenocarpic fruits are obtained with pears and apples when frosted shortly after or during the flowering stage, and while fruits of these species have occasionally been reported to be set with auxin sprays, the auxin treated fruits drop from the tree during development (130, 131) except in cases where frost has occurred during or just after flowering [van Stuivenberg (160); Roberts & Struckmeyer (139)]. The Bartlett pear which sets a good deal of parthenocarpic fruits naturally can be effectively set with auxin sprays in some localities (50, 68).

The single seeded tree fruits are usually neither parthenocarpic nor responsive to fruit set sprays, with a few exceptions. One exception is the apricot under conditions of frost (36, 37). The use of auxin to prevent dropping after frost will be discussed under the section on fruit drop. Another possible exception is the avocado which sometimes sets fruit parthenocarpically and will do so with 2,4-D (157), but in this case the fruits fail to develop to full size.

The failure to develop to full size is a frequent stumbling block to the effective use of auxins in fruit set. Several tree crops can respond to auxin treatment with fruit set, but the fruits are capable of only partial development and then are dropped from the tree. Examples of this are pears set with naphthoxypropionic acid (131) or with α-2,4,5-trichlorophenoxypropionic acid (12), apples set with 2,4-D, β-naphthoxyacetic acid (162) or α-phenoxypropionic acid (131), and olives with 2,4-D (73). The same limitation applies to hops (143) which set fruit with auxins, but the fruits then drop from the vine.

One treatment to obtain set in a tree fruit which would normally respond poorly to auxin is reported by Degman & Batjer (47). Pears, as already pointed out, are prone to be thinned by auxins rather than set; this is especially true with Anjou as contrasted with Bartlett pears. Reports of success with α-2,4,5-trichlorophenoxypropionic acid on Bartlett (50) led to repetitions of these tests with other varieties by Degman & Batjer (47)

who found that applications of this auxin to Anjou pears in August or September would result in fine increases in set the following spring. With the knowledge available as to the persistence of auxins in plants, it becomes very difficult to say whether such effects as these and other long term effects on citrus fruits to be discussed in a later section are due to the auxins themselves or to the aftereffects of the applications such as changed nutrition or growth.

Among the small fruits, auxins show promise of usefulness in the setting of grapes (169, 170, 172). Flowers of Black Corynth and Thompson Seedless grapes usually set poorly unless the stems are girdled—a property which these grapes share with some pears. The need for such an expensive operation can be considerably alleviated by spraying with *p*-chlorophenoxyacetic acid or benzothiazole-oxyacetic acid. The best set and fruit size is obtained when both the auxin and the girdling treatments are used.

With regard to seedlessness, when auxins are used to set fruits in the absence of pollination the fruits are seedless; and in many instances where pollination has occurred prior to treatment the ovules abort, resulting also in seedlessness. In some cases, however, normal seeds can develop after auxin application, and this property has been utilized by plant breeders for the improvement of fruit set when difficult crosses are being made. Burrell & Whitaker (23) first described the procedure using muskmelons. Pastes of indoleacetic acid were applied to the female flowers at the time of pollination, and successful fruit set and seed production were doubled. The effect is a consequence of the prevention of abscission of the flower and perhaps a longer persistence of the style during the period of pollen tube growth. The same procedure with this and other auxins has been utilized for lily (55), lima beans (133, 175), potatoes (176, 184), blueberries (45), snap beans (124), petunia (58), pear, and apple (44).

Another use of auxins in fruit set is the setting of berries on holly (32, 61), and Christmas cherry (177).

On the basis of fruit-set effects.—In the natural course of fruit set, either pollination causes a burst of growth hormone production (119, 120) or the natural level of hormone in the ovary is very high (69) and parthenocarpic fruit-set ensues. The presence of large amounts of growth hormone at fruit set appears to be general, and the fact that synthetic auxins can substitute for pollination in many instances suggests that auxin is itself responsible for fruit set. As pointed out by Nitsch (128), this growth hormone may arise from the pollen, from the tissues of the ovary, or from interactions between the pollen and the ovary. Each of these sources has been demonstrated in various plant materials (93, 109, 128). It is logical to assume that the application of synthetic auxins to bring about fruit set simply provides the hormonal stimulus for fruit growth that would normally occur with pollination. The molecular requirements for activity in fruit set have been found by Osborne & Wain (130, 131) to correlate very precisely with the requirements for auxin activity. This picture is

clouded somewhat by the occasional reports of substances which are clearly not auxins themselves and yet cause fruit set. Various phthalamic acid derivatives are in this category (81), but after the original report observers have noted that the apparent stimulation of fruit-set might have been a consequence of a retardation of abscission of the flowers. Another report (141) indicates fruit setting by 4-phthalimido-dimethylpyrimidine which cannot be an auxin for lack of a carboxyl group. Gibberellin has also been reported to cause set (178). With these few reservations the generalization of Nitsch (128) appears valid; fruit setting by growth regulators is a function of their auxin activity.

The limitation of auxin effectiveness in setting fruits to certain groups of plants suggests that in general there might be an additional factor required for fruit set. Steward & Simmonds (154) have made the interesting implication that, in addition to the auxins, growth stimulants of the sort that they have extracted from parthenocarpic banana fruits may be involved in the commencement of fruit growth. The species which do not respond to auxin applications with fruit set may be lacking in such additional factors.

In addition to the stimulation of fruit development, auxins applied at fruit set act to retard or delay abscission of the flower. This additional factor for the encouragement of fruit set provides a means for the improvement of pollination by plant breeders.

Fruit Thinning

From the time that it was first noted that naphthaleneacetic acid applied to apple flowers failed to set the fruits and in fact caused a decrease in the fruits' set (22), the fruit thinning effects of this compound have been considered to be an auxin effect (6, 8, 93). It is extremely curious, however, to note that the successful thinning of fruits appears so far to be an exclusive property of naphthaleneacetic acid among the auxins. Derivatives of this acid are effective also, the amide being very widely used because it provides thinning effects with less damage to the foliage (as flagging and epinasty) than the free acid. While we have considered this action to be an auxin effect (93), the only reports to date of effective thinning with other auxins have been concerned with flower thinning, as for example the thinning of grapes with *p*-chlorophenoxyacetic acid (172a) and the thinning of pears with α-2,4,5-trichlorophenoxypropionic acid (68). One other material which has been found to be effective in fruit thinning is chloro-IPC (chloroisopropyl-*n*-phenylcarbamate) on peaches (82, 110). This is not an auxin. Its effectiveness as a thinning agent is offset by the fact that it depresses tree vigor and results in the occurrence of deformed and prematurely ripening fruits, especially on trees which are weak or low in nitrogen (21). Chloro-IPC is not presently being used for fruit thinning. In contrast to fruit thinning, quite a number of materials are effective in flower

thinning. Included among these chemicals are cresols and phenols [see (8)], N-1-naphthylphthalamic acid (52), and the several auxins mentioned already.

At present there is wide use of naphthaleneacetic acid, and especially of its amide, for thinning of apples and on a more limited scale for the thinning of peaches. It shows promise of becoming accepted for the thinning of olives (74), pears (68), and possibly grapes (172). The amide form is used at higher concentrations but has distinctly less formative effects on the foliage.

Since only one auxin is known to be effective in fruit thinning, we should be cautious about considering the effect to be an auxin response. One is tempted to think about it as such since the principal means by which the thinning is accomplished appears to be through the abortion of the embryo of the young fruit (123, 163), an event commonly induced by auxins (161). On the other hand, ovule abortion is not unique to the auxins by any means.

Another characteristic of fruit thinning with naphthaleneacetic acid is a markedly greater concentration sensitivity than in most auxin applications. In most of the uses of auxin, the concentration-response curve is roughly logarithmic within a quantitative range, the singular exception being the application to prevent preharvest drop of fruits which is almost an all-or-none response (93). In comparison, fruit thinning is interesting in having a very steep response curve. For example, concentration curves such as those published by Alderman (2) show that 25 p.p.m. of naphthaleneacetic acid will completely thin some apple varieties and not thin others at all. Those unthinned by 25 p.p.m. may be completely thinned by 50 p.p.m. These sample data illustrate the problem of the orchardman who must somehow find the delicately poised level of application which will partially thin fruits and not remove excessive quantities.

The fears of the practical apple grower concerning the dangers of overthinning are partly tempered by the fact that in apple trees with biennial bearing tendencies the removal of too many flowers in one year will have the happy consequence of increasing the amount of flowering the following or off year.

It is well known that the chemical thinning of apples is limited to the first three weeks after full bloom. After that time the embryo in the seed has launched into its phase of rapid growth and appears to be resistant to damage by auxin application (107). The later the spray treatment, the less the effect on thinning. Hand in hand with the changing effectiveness in thinning, is the changing effectiveness in promoting the production of flowers for the following year. The interactions of thinning and of flower formation are well illustrated by Figure 1, in which early applications of naphthaleneacetic acid caused very effective fruit thinning, and at the same time permitted large gains in the flower formation for the following year.

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Later applications were less effective for each of these two functions to a proportional degree. Flower formation is inhibited rather than promoted if the naphthaleneacetic acid is applied at too high concentrations or if the spray is applied after the termination of June drop—about four weeks after bloom (87, 121).

The effectiveness of the thinning spray may be markedly altered by conditions leading to differences in the vigor or color of the tree (121), a fact

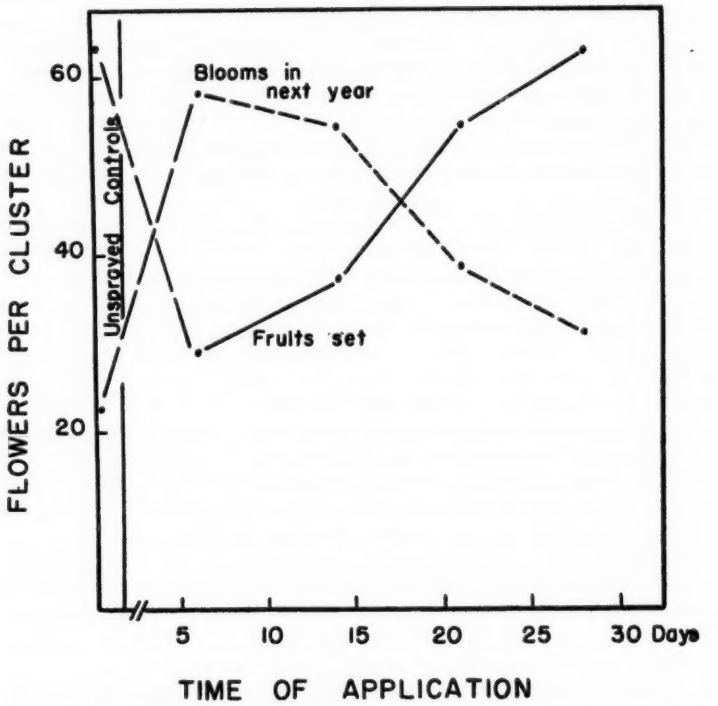

FIG. 1. The influence of a fruit thinning spray of naphthaleneacetamide on (*a*) the numbers of fruits set per 100 clusters, and (*b*) the numbers of flowers initiated for the following year. Data of Alderman (2) for three concentrations and five apple varieties.

that leads growers to spray trees which show a lighter foliage color much more lightly. While experiments with different nitrogen levels have failed to show an influence on the effectiveness of sprays on apples (153), growers generally consider the nitrogen influence to be real.

By the use of radioactive tagged naphthaleneacetic acid, Harley *et al.* (70) have performed some ingenious experiments to analyze the thinning treatment. They found a decidedly better entry of the auxin into leaves after a low temperature experience and established that this correlated with a greater effectiveness. They also noted a greater penetration into the lower side of the leaf than into the upper and recommend spray methods that would specifically wet the lower surfaces. Detergent carriers have a mild fruit thinning action in addition to increasing the penetration of the naphthaleneacetic acid.

A deterrent to the general use of auxin fruit thinning sprays has been a growing concern among growers that naphthaleneacetic acid and, to a lesser extent, naphthaleneacetamide cause a gradual decline in the vigor of the apple trees. This decline may be more pronounced among the early ripening varieties such as Transparent and Duchess (21).

On the basis of fruit thinning.—In the last review of this subject (93) the thinning of flowers and fruits was attributed to three actions of the naphthaleneacetic acid: (*a*) the prevention of pollination; (*b*) the induction of embryo abortion; and (*c*) the alteration of the auxin gradient across the abscission zone causing abscission. These explanations might be re-examined. The first, the prevention of pollination, is the action by which flowers are thinned. Auxin solutions applied to flowers cause a state of incompatability in the styles (108), an inhibition of pollen germinability and growth (1), and a burning of floral parts receiving the spray (74). The cresols and phenols commonly used for flower thinning have the same flower-burning action. The second action, the induction of embryo abortion, appears to be the main action in the thinning of fruits. After a thinning spray of naphthaleneacetic acid there typically occurs a great increase of embryo abortion which is followed in turn by a burst of fruit abscission (108, 163), the abscissing fruits being the ones in which such abortion has taken place (163). In the earliest stages of fruit growth the endosperm is most readily aborted by auxin (161), and its demise is soon followed by collapse of the dependent embryo. The embryo is in a very elementary stage of development before and during June drop of apples, and in this stage the embryo itself is evidently quite susceptible to abortive influences. After the termination of June drop when the embryo enters a stage of rapid growth, thinning can no longer be obtained with naphthaleneacetic acid—— a fact consonant with the increasing resistance to abortion of older embryos (138, 161).

After the application of the thinning spray, the drop of apple fruits is arrested in most instances (123, 153, 159, 163), and growth of all of the fruits is markedly retarded (163). This is followed by the occurrence of aborted endosperms and embryos noted already, and abscission follows.

The concept that the naphthaleneacetic acid applications bring about fruit thinning by altering the auxin gradient across the abscission zone

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such as to increase the proximal auxin supply in proportion to the distal appears to be unlikely. The immediate retardation of fruit abscission does appear to be a direct effect of the auxin upon the abscission zone——a fact demonstrated by Murneek & Teubner (123). They removed apple fruits by hand after the thinning spray, leaving the pedicels on the trees, and the pedicel abscission was still retarded by the auxin spray. In general, auxin treatments which do promote abscission do not cause inhibitions before the promotions begin. This is clear from time curves of abscission in the bean test (16). Finally, the lack of any thinning action by sprays applied after June drop also discounts the concept of an auxin promotion of abscission by changing the gradient (163).

It is quite possible that the promotion of abscission may be more involved than a simple reduction of auxin supply from the endosperm. The interesting suggestion has been made by Teubner & Murneek (163) that ethylene production by the aborting fruits may play a part in the abscission. They found that dropped fruits emit such a gas as indicated by tomato epinasty tests.

Mention should be made of the suggestion by Struckmeyer & Roberts (159) that thinning may be a consequence of increased set in response to the auxin spray, followed by greater competition between the fruits for nutrients. It is true that in many cases the auxin spray is followed by a brief period of decreased fruit abscission, as cited above, but there are also cases in which this clearly does not occur and yet thinning follows (12). Also, the retardation of abscission should not be confused with increased fruit set. It is quite true, nevertheless, that fruits with a nutritive advantage such as the king or terminal fruits in an apple cluster are much less susceptible to thinning sprays than other fruits on the cluster. Nutrient competition is keener among the smaller fruits, and these are the ones most prone to abscise.

It is very curious that thinning sprays act principally through the foliage. Harley *et al.* (70) have correlated the effectiveness with the degree of penetration into the foliage of apples. Hartmann (74) has obtained fine thinning of olive fruits when naphthaleneacetic acid was applied to the foliage, but no thinning when applied to the fruits themselves. Peaches are quite unresponsive to such sprays during the first three weeks of flowering ——when there typically is no foliage; only later are thinning sprays effective (79), and at this time leaf buds have opened. If our concept of embryo and endosperm abortion is entirely accountable as the cause of fruit thinning, then one would not expect this to be the case.

The lack of effectiveness of other auxins in thinning and the locus of its effective application suggest that some subtle factor of the movement of naphthaleneacetic acid from the foliage to the ovule may be involved in the thinning mechanism. Alternatively there could be a mobilization effect of the auxins on the leaves which deprives the young fruits of substrates for growth, resulting in embryo abortion and abscission.

Fruit Drop

The control of the abscission of fruits by application of auxins is perhaps the most clear-cut and uncomplicated use of auxins in agriculture. The action is clearly attributable to auxin itself. The underlying physiological function involved——inhibition of abscission——can be identified and the effects of auxin upon this function are recognized. These facts form a reasonable basis for understanding the effect on fruit drop.

The use of auxins for this purpose has been reviewed elsewhere (6, 93, 166), and the situation has not been markedly altered since.

The inhibition of abscission by auxin has been recognized for many years. When auxins were first applied to apple trees for the prevention of preharvest drop (62), the way was opened for the general use of naphthaleneacetic acid by orchardmen. This auxin has several limitations, however. While it gives a quick inhibiting action, its effectiveness persists for only one or two weeks, and it is not effective on some of the commonest American varieties. 2,4-D was taken up for use on a limited number of varieties of apple. While its effects were longer lasting, it causes growth deformities; and it is not effective on all varieties. 2,4,5-trichlorophenoxyacetic acid was then found to be effective for longer periods of time and to be widely effective, surpassed only by α-2,4,5-trichlorophenoxypropionic acid. This last material encompasses many advantages: wide effectiveness among fruit varieties, persistence of effects for long periods, and the ability to heighten the red coloration of apple fruits.

The use of fruit drop sprays has led to the development of a new problem to the pomologist, the problem of fruit storage after the hastening of ripening and softening by the added auxin. Prior to the early 1950's the problem was minor as naphthaleneacetic acid has little ripenening effect, and being applied shortly before harvest meant it had only a short time in which to affect ripening processes. Since that time, however, the increasing use of the phenoxy acids has made the problem acute. These materials are often sprayed onto the trees a month or more before fruit harvest, producing marked coloration changes and distinct and undesirable changes in the softening and keeping quality of the apples.

The amide of the α-2,4,5-trichlorophenoxypropionate appears to have the strongest ripening effects of the chemicals used (150) along with being the most effective preventor of drop (51, 53), and the propionic acid is more detrimental in hastening ripening than the acetic derivative (114, 150, 152). The ripening effects are worse on exposed than on shaded fruits (122), on high nitrogen than on lower nitrogen trees (150), on summer than on fall varieties (105, 122), and worse in the eastern states than in the central and western states (9, 152). The higher the concentrations of auxin applied and the earlier the application, of course, the worse the ripening effect.

Two possible means of meeting the ripening problem have appeared: (*a*) the incorporation into the auxin solution of an additional material

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which might counteract the auxin stimulation of ripening; or (*b*) the restriction of the spray treatments to periods of time sufficiently close to harvest time that fruit ripening will not be increased. The first means was the clever suggestion of Smock *et al.* (149). They used maleic hydrazide to limit the ripening effect. This procedure has met with limited success. The maleic hydrazide markedly lowers the respiratory and softening stimulations following naphthaleneacetic acid spray, is less effective following α-2,4,5-trichlorophenoxypropionic acid spray, and after 2,4,5-trichlorophenoxyacetic acid it lowers softening only about 50 per cent (150). Another limitation appears to be that the effects of the inhibitor persist for less time than the durable trichlorinated auxins (34, 150). The second possibility for meeting the ripening problem appears to be unlikely to be accepted by orchardmen as it means giving up the extra fancy color achieved with the early application of the auxin spray.

It is interesting to see that the continued improvement of a chemical application to agriculture should bring with it the continued intensification of a new problem.

Turning to the control of fruit drop in species other than apple, the citrus fruits are controlled widely by auxin sprays at the present time. The use of auxins for these fruits has remained essentially unchanged since the appearance of the last review of the subject (93). 2,4-D and 2,4,5-trichlorophenoxyacetic acid are used on oranges and lemons providing the combined beneficial effects of limiting preharvest fruit drop and also increasing the fruit size. One of the most curious aspects of this use of auxin is that the chemical is applied to citrus species even before the flowers open in order to influence abscission and size of the fruits which will ripen as much as seven months later.

Apricots offer a serious fruit drop problem. In many areas considerably more than half of the fruits drop from the trees before ripening. Early attempts to prevent drop with naphthaleneacetic acid (78) only partially retarded the process. Experiments designed principally to study the effects of 2,4,5-trichlorophenoxyacetic acid on fruit enlargement led to the finding that this auxin could provide good control of the drop of Royal apricots (35). Even better control could be obtained with this compound on Tilton and Stewart apricots (38). These fruits offer a curious physiological picture. While some varieties such as Stewart apparently drop fruits because of abortion of the embryos and the presumed consequent decline of auxin production in the fruits, Tilton apricots drop even with apparently healthy embryos and presumably a normal auxin complement (38). Nevertheless, the Tilton fruits yield the most complete response to auxin treatments for prevention of drop. The dramatic extent of the effect is illustrated in Figure 2. The figure also illustrates the long duration of the auxin action——in this case in excess of 14 weeks.

In addition to the species already mentioned——apple, apricot, orange, and lemons——control of preharvest drop has also been reported for pears (11), prunes (72), almonds (144), and cotton (15).

On the basis of fruit drop effects.—As already mentioned, the action of auxin in this use appears to be a clear case of the inhibition of abscission ——a property identified with auxins since 1936. The applications of auxin are most effective when made to the fruits themselves, as would be expected (62), though auxin absorbed through the leaves is also effective (10). In the case of the apple, auxin production by the seeds declines rapidly as the fruit matures (108) and the resulting stimulation of abscission can be

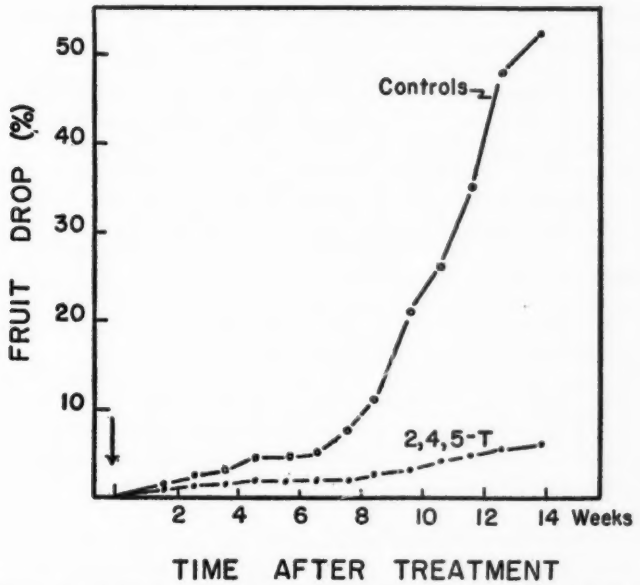

FIG. 2. The inhibition of fruit drop in apricots by the application of 25 p.p.m. 2,4,5-trichlorophenoxyacetic acid. The auxin was applied at the time indicated by arrow [Data of Crane (38)].

averted by application of auxin spray to the fruit. Seeds being the principal source of auxin to the fruit (106), apples with fewer seeds are more liable to drop (75).

The relative effectiveness of different auxins varies between varieties making it difficult to say flatly whether activity in the control of fruit drop

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correlates with auxin activity. Some dramatic differences in effectiveness of auxins between apple varieties have been described on a quantitative basis by Edgerton & Hoffman (51). It is interesting to note that in general the effectiveness of 2,4-D is highly variable between apple varieties, and 2,4,5-trichlorophenoxy acids show the greatest activity and the most uniform response between varieties [see also (111)]. Day & Erickson (46) have examined a complete array of chlorinated phenoxyacetic acids in retarding lemon petiole abscission, and their conclusions are quite similar to those from the apple work.

The remarkable duration of effectiveness of the 2,4,5-trichlorophenoxy acids suggests that these auxins are very persistent in the plant. It may be recalled that they are broken down very slowly by soil microorganisms (5), and the same durability may apply in plants (93). A feature of these compounds which may well contribute to their persistence is the strong adsorptive qualities they show (94). As a matter of speculation, it is reasonable to think that very strong adsorption of these auxins to surfaces within the plant would make them more resistant to breakdown.

The abscission of fruits as a consequence of frost can often be averted by auxin treatment. In 1943 it was observed simultaneously with pears (160) and with apples (139) that fruit drop normally occurring after frost could be prevented with naphthaleneacetic acid. These observations were apparently overlooked for a number of years until Crane (36, 37) reported that the same could be done for apricots, entirely preventing the enormous losses that normally follow frost. Since that time the usefulness of auxins for frosted pears has been confirmed by Griggs *et al.* (67).

The observations of Crane (37) indicate that the auxin treatment does not prevent frost damage to the fruits themselves for the surfaces of sprayed fruits blister and discolor in the same way as control fruits. Furthermore, many of the embryos within the sprayed fruits abort, though fewer than in the control frosted fruits. The abscission of the fruit is avoided, however, by the supplementary auxin supply. The action of the auxin through the simple prevention of abscission is indicated by the fact that auxin treatment after the frost is essentially as effective as treatment before frost. Of course, the persistent trichlorinated phenoxy acids are the most effective auxins in this use, persisting in effectiveness until the fruits are harvested (37, 67).

The question frequently arises as to why the application of naphthaleneacetic acid early in the season causes fruit thinning while the same material at essentially the same concentration applied late in the season prevents fruit drop. The explanation is quite straightforward within the limits of physiological understanding: (*a*) the auxin causes a retardation of abscission in both the young fruits (with a few exceptions) and the old fruits; (*b*) the embryos in the young fruits are immature up to four weeks

after bloom, and immature embryos are more susceptible to abortion by auxins (138, 161). Therefore, the early auxin application results in their abortion, and the fruits subsequently abscise. The later application finds the embryos quite resistant to such abortive influences; therefore, the only effect resulting from the auxin application is the retardation of abscission.

Fruit Quality

Auxin applications are useful for modifications of several aspects of fruit quality including fruit size, earliness of ripening, color, and storage quality. These several aspects are frequently interrelated.

During phases of fruit growth which are limited by the auxin supply the application of auxins will increase the growth rate of the fruit. Under some circumstances this will result in the achievement of the state of termination of growth and ripeness earlier than would normally occur ——as in the case of fig and apricot——or the continuation of growth for a longer period, delaying the ripening events——as in the case of citrus fruits and pineapple. At the same time that growth responses are evoked, changes in the types of metabolism certainly occur; and these are reflected in changes in the development of such ripening processes as coloration and softening. It is reasonable then to consider these various effects on quality together as they shade so closely into each other.

As an aid in discussing the alterations of fruit size, we may recognize at least two different patterns of fruit growth curves represented schematically in Figure 3. A simple sigmoid growth curve describes the fruit enlargement in many fruits with large numbers of seeds as in pineapple, apple, strawberry, and tomato. The application of auxin to the pineapple, for example, causes a continuation of the grand period of growth extending the sigmoid geometry over a longer span and yielding a larger fruit at a later harvest time (Fig. 3A). A second type of growth curve is shown in Figure 3B where the sigmoid function is interrupted by a retarded growth rate after the fruit has reached about half size. This type of curve is common to the fig, prune, cherry, currant and apricot. If auxin is applied to the fig during the retarded growth phase, the full rate is restored and the fruit reaches full size or slightly more than full size with an earlier ripening date. A quantitative variation of the second type of growth curve occurs in the apricot, in which the retarded growth phase may last for six weeks. With applications of low concentrations of 2,4,5-trichlorophenoxyacetic acid, an intermediate growth rate is obtained until the end of the lag period. Applications of higher concentrations of the auxin can restore full growth rate, and of course this leads to the greatest increases in earliness and final fruit size (40a).

There are many variations of this scheme. Peaches and mangoes, for example, are caused to ripen quickly (88, 174), and any stimulation of

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growth rate after the auxin application is apparently cut off by the ripening process. The fruits finish off with a smaller final size. Tomatoes and strawberries which have the first type of growth curve (Fig. 3A) may be stimulated in growth during brief periods of the middle and early sections of their growth curves, respectively, resulting in increased final size (147, 187). The available evidence suggests that auxins increase citrus growth rates through the entire fruit growth period (57), a fact which may be explained on the basis that the auxin caused a reduction in the total numbers of fruits on the plant; the over-all increase in growth rates then appears to correlate with the smaller fruit load on the plant. The same may be true of the grape (171). Another variation is found in the prune. This fruit has the second type of growth curve, and auxin application during the growth lag causes the same type of response as shown in Figure 3B for

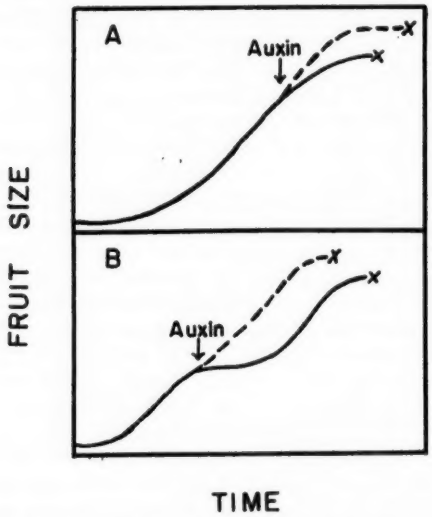

FIG. 3. Schematic representation of the effects of auxins on fruit growth, ripening and final size. Arrows indicate times of auxin application, and crosses indicate times of ripening.

the fig with earlier ripening and increased size; however, auxin applied after the lag phase hastens ripening directly with a consequent decrease in fruit size (71). In addition to the species already mentioned, gains in fruit size have been obtained in the blackberry (20, 109a, 188).

Girdling, or ringing the bark, increases over-all growth rates of fruits.

An interesting comparison between girdling effects and auxin effects on fruit growth in apricot was made by Crane & Campbell (42). Girdling of branches resulted in an increased growth rate during the entire growth period of the fruits, whereas the auxin application only increased growth after pit hardening—which period coincides well with the period of retarded growth rate shown in Figure 3B.

The basis of the auxin effects on apricot fruit size has been examined in some detail by Crane's group. They found that the gain in size was accountable as an increase in cell volume. Fruits of the genus *Prunus* terminate cell division activities soon after pollination, and this situation is apparently unchanged by the auxin treatment (19). Cell size is strongly increased, however, resulting in increases of 50 or 60 per cent in cell volumes. This swelling is associated with large amounts of nuclear division and proliferation of the chromosomes resulting in enormous amounts of endopolyploidy (19). Analyses of the fruits indicate that the fruit weight increases are principally water increases, for the dry weight of the fruits treated with auxin are not much different from untreated ones (41). There is an increase in sugar content per fruit which leads to the suggestion that the auxins cause a mobilization of soluble carbohydrates in the fruits.

With respect to the retarded growth phase of fruits, Nitsch (128) suggested that these retardations may be a consequence of a diversion of growth activity (or growth hormone, perhaps) to the embryo or endosperm. This suggestion may also be applied to the occurrence of June drop in apples which takes place during the period of rapid growth of the endosperm (107). Examination of the effects of the auxin applications on growth of the embryo and endosperm of the apricot failed to reveal a suppression of these organs which might have helped to account for the rise in growth of the fleshy parts of the fruit (43).

Auxin effects on fruit coloration are most pronounced in the apple, and as pointed out earlier, the current use of the apparently persistent 2,4,5-trichlorophenoxy acids for the prevention of fruit drop has greatly heightened the red pigments in apples over most parts of the United States. It is curious to note that applications of maleic hydrazide to retard the maturation of apples which have received α-2,4,5-trichlorophenoxypropionic acid selectively retards the softening responses to the auxin without apparently retarding the coloration increases (149).

In addition to the detrimental effects of fruit drop sprays upon apples, one other storage effect should be noted: the use of auxins to improve the storage quality of citrus fruits. Considerable losses of citrus fruits in storage occur due to "black button," the result of invasion by the mold *Alternaria*. This pathogen enters through the abscission zone of the fruit, and its entry can effectively be prevented by the retention of a healthy pedicel piece, or button, on the fruit. If the button deteriorates or abscises,

the rot may readily spoil the fruit in storage. The problem is worse in the case of ethylene treated fruits——a common treatment for the improvement of coloration of lemons and oranges——for ethylene markedly increases the abscission of buttons. The retention of the button in a healthy condition has been achieved by dipping the fruits in 2,4-D or 2,4,5-trichlorophenoxyacetic acid (56, 57, 155, 156). Some control of the rot is obtained from applications of auxin to the trees to control fruit drop and ripening (156), but treatment of the fruits after harvest is more effective and is widely used in citrus packing plants.

Discussion

The enormous diversity and complex requirements for the useful applications of auxins in the modification of flower and fruit activities lead us to attempt some generalizations and deductions.

The wide variety of effects obtained with auxin applications is hardly compatible with any concept of a simple or single action of these chemicals on plants. The long list of auxin effects upon flower initiation, fruit set, fruit thinning, abscission, growth, respiration, pigmentation, and even pathogenicity is discouraging to any attempt at a simplified explanation. Much more logical is the concept that auxins are biologically very active materials, and they will not only control simple cell growth (perhaps through some precise action upon the cell wall), but also they can interfere with many metabolic activities. They have strong adsorptive properties and will attach themselves to many different sites in the plant——on the surfaces of various cell walls, membranes, interfaces, and enzymes. Most of these attachment points will probably not be the hypothetical activity sites for auxin action, and so perhaps most of the actions of these chemicals on plants may not be a direct consequence of their auxin activity in the strict sense used by growth physiologists. Mention has been made of the diverse array of compounds other than auxin which can bring about effects on flower initiation, fruit set, and fruit thinning. It would seem a reasonable suggestion, therefore, to consider these "auxin effects" as being a consequence of exposure of the plants to chemicals of strong biological activities common to auxins but not limited to them.

One might comment also on the curious specificity of the loci of auxin application and effect. When auxins are applied to plants in the field at concentrations which are useful for the control of flowering or fruiting processes, there seems to be a great absence of evidence that these auxins move about in the plant. Application at concentrations approaching herbicidal levels certainly results in movement, and even at concentrations which cause the gentler responses of telemorphic or other gross morphological changes (epinasty and rooting included), the auxins are moved about. In fact a great deal is known about the characteristics of such movement

(93). But consider how localized is the effect of an application of 25 p.p.m. of naphthaleneacetic acid to apples for thinning (10). Applications of 10 p.p.m. of *p*-chlorophenoxyacetic acid to one part of a grape cluster for thinning will not even alter the other parts of the same fruit cluster (171). It may be a mistake to consider the fruit responses to auxin applications to the foliage as being a consequence of the absorption of the auxins through the foliage and the subsequent transport to the fruits. In some instances it may be more accurate to consider the dilute chemicals as having their effects at the actual locus of application and the response of organs farther away as being due to secondary effects such as resultant changes in nutrient distribution.

Speculating further about the locus of auxin effects, it is startling to find that auxin applications to apricot trees have quite different effects on the fruits than treatments applied directly to the fruits themselves (115). The foliar application results in a depression of respiratory activity in the fruits, whereas dipping the fruits in the auxin solutions results in an acceleration of respiration. Also the hastening of ripening following foliar treatment is not apparently obtained after fruit treatment. These findings are difficult to rationalize into an interpretation involving the translocation of the auxin from the leaves to the fruits.

One more comment on locus specificity might be made. The action of these chemicals varies enormously with the exact stage of development of the plant, flower, or fruit at the time of application. Naphthaleneacetic acid applied to apples in the first three weeks after bloom thins the fruits and consequently promotes flower initiation (Figure 1); application of the same concentration one week later has no effect on thinning and inhibits flower initiation. Again, application of auxin to plums at the time of the growth lag in the fruits increases growth and fruit size; the same treatment one week later decreases growth and fruit size.

There are moments of critical change in the development of fruits with respect to auxin responses. One of these may be the time of commencement of embryo development. In fleshy fruits such as the apple, the embryo begins rapid development three to four weeks after bloom, the time after which auxins will not thin fruits. In stone fruits this is about the time of pit hardening, the time after which auxin applications lose effectiveness, for fruit thinning or fruit size. Another moment of critical change is certainly the onset of the climacteric respiratory peak. Auxins applied to fruits which have begun to ripen——as in red tomato fruits or ripe apple fruits——have no promotive effects on respiration or ripening where prior to this point good respiratory or ripening responses can often be obtained (54, 115, 151).

In conclusion it seems pertinent to point out one of the commonest pitfalls confronting the researcher in the field of auxins. With the applica-

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In conclusion it seems pertinent to point out one of the commonest pitfalls confronting the researcher in the field of auxins. With the applica-

tion of these compounds to growing plants so many different types of responses are possible, and the conditions for changing the responses from one type to a completely different or even opposite one are so subtle, that extreme caution is required in drawing conclusions or making recommendations. Before an auxin treatment is accepted for commercial use it should be widely tested. The limitations as well as the virtues of its action should be well understood.

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HERBICIDES[1,2]

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Three reviews on herbicides have already appeared in this series, Norman *et al.* (219), Blackman *et al.* (37) and Crafts (65). During the four years since the last review approximately 10,200 papers on chemical weed control have been covered by *Weed Abstracts* (330). Most of these papers relate to work on the evaluation of herbicides under field conditions and have as their object the development of practical recommendations. Many are interim reports published in the Proceedings of Weed Control Conferences. Recent reviews dealing with different aspects of this practical side of the subject are as follows: (*a*) agriculture: Åberg (5), Ennis (82), Frankton (98), Rademacher (240), Russell (265), Shaw, (272), Stryckers (294), Templeman (301), van der Zweep (315), Woodford & Kasasian (348) and Zonderwijk (356); (*b*) horticulture: Payrebrune St. Sève (232) and Stryckers (293); (*c*) aquatic weeds: Chancellor (57), Härdtl (126), Mackenthun (191), Parker (231) and Schofield (269). In addition several books on chemical weed control have been published: Melnikov *et al.* (200), Riepma Kzn (253), Tukey (311) and Wehsarg (333).

A single review can no longer do justice to this vast subject. This chapter will, therefore, be concerned chiefly with the principles relating to the mechanisms of action of herbicides and not with the technology of their

[1] The survey of literature pertaining to this review was completed in September, 1957.

[2] The following abbreviations will be used: ATA for 3-amino-1,2,4-triazole; CDAA for 2-chloro-N,N-diallylacetamide; CDEC for 2-chloroallyl-N,N-diethyldithiocarbamate; CIPC for *iso*propyl-N-(3-chlorophenyl)carbamate; 4-CPA for 4-chlorophenoxyacetic acid; 2,4-D for 2,4-dichlorophenoxyacetic acid; 4-(2,4-DB) for γ-(2,4-dichlorophenoxy)butyric acid; DNBP for 2-*sec*butyl-4,6-dinitrophenol; DNC for 2-methyl-4,6-dinitrophenol; EPTC for ethyl-N,N-dipropylthiolcarbamate; IAA for 3-indoleacetic acid; MCPA for 4-chloro-2-methylphenoxyacetic acid; 4-(MCPB) for γ-(4-chloro-2-methylphenoxy)butyric acid; 2-(MCPP) for α-(4-chloro-2-methylphenoxy)propionic acid; MH for maleic hydrazide; NPA for N-(1-naphthyl)phthalamic acid; 2,4,5-T for 2,4,5-trichlorophenoxyacetic acid; 4-(2,4,5-TB) for γ-(2,4,5-trichlorophenoxy)butyric acid; 2,3,6-TBA for 2,3,6-trichlorobenzoic acid; TCA for trichloroacetic acid; 2-(2,4,5-TP) for α-(2,4,5-trichlorophenoxy)propionic acid.

Where applicable, these are the abbreviations approved by the Weed Society of America (27).

[3] The authors are indebted to their colleagues in the Unit, in particular Dr. Daphne J. Osborne, Dr. Leonora C. Reinhold, and Mr. R. G. Powell, for help in the preparation of this review, and to Mrs. D. M. Holly for assistance with the literature references.

application. The gap between the applied and the fundamental, in so far as plant growth-regulating chemicals are concerned, has been bridged in a book by Leopold (181), while reviews on the modes of action of the whole range of phytotoxic chemicals have been written by Crafts (66, 69), Currier (71) and Woodford (347).

Spray Retention

The first step in the operation of killing weeds with herbicides is the application of the chemical to the plants. If the entry of the herbicide is mainly via the foliage, then the amount of chemical that is retained by the plants is of primary importance as it determines the dose applied. Numerous data have been published on the retention of sprays by a range of species and the influence of such factors as stage of growth, variety of crop, volume of spray applied, formulation and surface tension of spray solution [Blackman *et al.* (34); Hellqvist (134); Woofter & Lamb (349)].

Blackman *et al.* (34), investigating five species with contrasting habits of growth, showed that the differences in retention between species could be large or small according to the stage of growth and the combination of spray output and surface tension selected. In the same experiments the equi-effective concentrations of several herbicides were determined. For MCPA (4-chloro-2-methylphenoxyacetic acid), 2,4-D (2,4-dichlorophenoxyacetic acid) and 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) the absolute amount deposited on the shoot was of primary importance in determining the phytotoxic effects. With the contact herbicide DNBP (2-*sec*butyl-4,6-dinitrophenol) more was required when the retention was low while for some species the enhanced phytotoxic effect caused by lowering the surface tension could not be ascribed to the change in retention.

The orientation and degree of pubescence of leaves [Ennis *et al.* (84)] and growth in shade prior to treatment [Dorschner & Buchholtz (77)] also influence spray retention. Ennis *et al.* observed by high speed photography that aqueous droplets bounced off the leaf surfaces of many species, and detailed studies of this phenomenon have been made by Brunskill (52). Using pea leaves, he showed that the proportion of droplets which bounced off was related to the surface tension of the spray and the droplet size; that it was influenced by the angle of incidence of the droplet and its velocity; but that varying the viscosity of the solution caused only small changes. In contrast, no such reflection of water droplets occurs from silicone or wax treated glass and Brunskill suggests that with leaves the reflection is associated with micro-roughness of the surface due to the exudation of granular wax. Electron microscope studies, particularly by workers at Iowa State College, have illustrated the variations that occur in the wax development on the leaf surfaces of different species at various stages of development (213, 267, 268). Further studies of this type could add much to our understanding of the behaviour of herbicidal sprays and the technique continues to be developed [Bradley & Juniper (42)].

Penetration into Shoot

The structure and chemistry of the plant cuticle in relation to the entry of plant growth regulators has been reviewed recently in detail by van Overbeek (316). The penetration of herbicides into plants has been studied by a variety of techniques. The simplest method is to wash the applied chemical off the leaf surface at various time intervals after application and to measure the resulting inhibition of growth. The assumption is then made that this inhibition is proportional to the amount of chemical that has been absorbed [Hauser (128); Standifer & Ennis (287); Barrier & Loomis (24)]. Caution is necessary in the interpretation of the results obtained by this method, for when Holly (143) washed leaf surfaces at different time intervals after application of a measured amount of MCPA and determined the quantity of herbicide present in the washings, he found a lack of correlation between the amount of chemical disappearing from the leaf surface and the subsequent inhibitory effect on the plant. Radioactive tracer techniques have been used in some instances (e.g. 283, 337). They can be very sensitive, but many workers who have used them to study the translocation and subsequent fate of herbicides inside plants have omitted to collect complementary data on entry. Another technique is concerned with the measurement of movement into and through isolated cuticle. Cuticle for such experiments has been prepared from leaf discs by fermentation using *Clostridium roseum* [Skoss (279)] and by a pectin polygalacturonase preparation [Orgell (224)]. Orgell (223) postulates three steps in penetration: (*a*) adsorption at the cuticle-spray solution interface; (*b*) absorption into the bulk of cuticle either by adsorption on internal surfaces or by solution within the cuticle; (*c*) desorption into the plasma membrane of the cell. He suggests that pH is important because it controls the polarity of the cuticle and often of the penetrant. Recently, however, he has indicated the part played by cations and has shown that under appropriate conditions equal absorption of 2,4-D can occur at high and low pH. The theory that 2,4-D is absorbed chiefly in the form of the undissociated molecule must thus be modified [Orgell & Weintraub (225)]. Lack of proportionality between absorption into tissues floating on or suspended in solutions and the concentration of undissociated 2,4-D molecules has also been reported [Blackman (32); Wedding & Erickson (331)]. On the other hand the results of Bennett (30) on the entry of 2,4-D, MCPA and IAA (3-indoleacetic acid) into cells of *Nitella* in culture were consistent with entry only as the undissociated molecules.

Many other factors which may influence the entry of herbicides into leaves have been studied. Although some variation has been found between species this has not been large, either with phenoxyacetic acids [Ashton (15); Holly (143)] or with MH (maleic hydrazide) (283). Weintraub *et al.* (337) found some differences between species in their absorption of 2,4-D but these were not correlated with susceptibility. Zukel and his coworkers (283) have found relative humidity as well as temperature to have

a very large effect on the absorption of MH. Blackman & Robertson-Cuninghame (36) found a lower absorption of 2,4-dichloro-5-iodophenoxyacetic acid when the plants were kept at a low light intensity after application. Behrens (26) showed that the phytotoxicity of the triethanolamine salt of 2,4,5-T decreased as the size of the droplets and the volume of spray were reduced. This he attributed to restricted absorption. Results obtained by Riepma Kzn (252) with DNC (2-methyl-4,6-dinitrophenol) are partly but not wholly in agreement. An interesting fact is that peas and other plants grown in soil that has previously been treated with TCA (trichloroacetic acid) or dalapon (2,2-dichloropropionic acid) retain more DNBP which penetrates more readily [Dewey *et al.* (76); Pfeiffer *et al.* (233)]. This effect is due to changes in wax formation and is of some practical importance where land has been treated with TCA or dalapon for the control of *Agropyron repens* prior to the planting of peas, and where it is intended subsequently to control annual weeds with a post-emergence selective herbicide.

The relation between molecular structure, penetration, and biological activity has been studied by a number of workers. Van Overbeek (318) citing work with a series of maleimides and amino phosphine oxides suggests that their biochemical activity within the cell is the same but that their physiological activity differs according to the lipophilic properties of the molecule which govern penetration and passage to the site of action within the cell. On the other hand Ross & Ludwig (263), investigating the phytotoxicity of an homologous series of N-*n*-alkylethylenethioureas found no evidence that toxicity depended on their ability to penetrate cytoplasmic membranes. A correlation exists with the oil-water partition coefficient, but there is a similar correlation with water solubility and probably with other physical factors. They do suggest, however, that once the chemical is inside the cell differences in lipoid solubility could be important in connection with internal accumulation. The role of lipoid solubility in penetration into leaves depends on the pathway of entry into the leaf and this is still the subject of dispute. Orgell (223) has postulated the steps in passage through cuticle outlined above. Against this Skoss (279) found no movement of water soluble formulations of 2,4-D and DNBP through isolated cuticle in which there were no stomata as contrasted with appreciable passage through cuticle with stomata. He indicates that herbicides dissolved in aqueous carriers enter primarily through stomata and only oils diffuse through the cuticle. Further experiments to test the universality of this important principle are obviously most desirable particularly as there is no evidence that isolated cuticles behave in the same manner as intact cuticles on living leaves.

All the herbicide which enters a leaf is not necessarily available for translocation, and Leopold (180) has summarized some interesting work which indicates that adsorption by leaves may differ markedly according to

the compound and species. He suggests that his results and the earlier work of Brian (47) indicate that adsorption on nonactive locations may determine the relative resistance of the plant. One such important location may well be the cuticle.

Availability in Soils and Entry into Roots

Availability to roots.—Most herbicides can be absorbed by plant roots and many of the newer compounds have little effect if applied only to the foliage. The factors influencing the availability of herbicides for uptake by the roots from the soil are therefore of great practical importance.

The chemical may reach the soil in which crops and weeds are growing as a result of: (*a*) a direct application to the surface by one of the many pre-planting or pre-emergence techniques used for selective weed control; (*b*) a post-emergence spray application which has not been intercepted completely by the foliage; (*c*) a post-emergence application of a granular formulation that is shed by the foliage [Danielson (73)]; (*d*) a residue in the soil resulting from earlier selective or nonselective application; or (*e*) contaminated irrigation water [Bruns (50, 51)].

Special cultivations may be employed to incorporate the herbicide into the soil, but usually the chemical has to reach the root system either by vapour movement or, more commonly, by leaching. The leaching of herbicides has been studied by many workers using a variety of techniques. In some instances chemical methods have been used to determine the fate of herbicides as in the experiments of Sund (295) with ATA (3-amino-1,2,4-triazole), Jensen & Larsen (154) with sodium chlorate, Hill *et al.* (140) and Birk (31) with monuron (N-(4-chlorophenyl)-N′,N′-dimethylurea) and Dowler & Baughman (78) with DNBP. More frequently, however, only bioassay methods have been employed.

Herbicides vary considerably in their ease of movement within soils and this cannot be predicted from their solubility or molecular size [Ogle & Warren (221)]. At one extreme are compounds, such as dalapon, which leach readily, as shown by the investigations of Holstun & Loomis (145) and at the other are compounds, such as the carbamates, which remain in the surface layers of the soil [Ennis (81)]. Resistance to leaching may enable a herbicide to be used selectively on a deep sown crop because the soil prevents the chemical from reaching the roots of the crop rather than because the crop is wholly resistant to the herbicide. However, the effectiveness of the herbicide is dependent on its ability to reach the germinating weed; see, for example, the work of Wiese & Dunham (343) on the use of carbamates for wild oat control. Naturally such selectivity is obtainable only if the weed seeds germinate solely in the soil layers above the crop seed. Many environmental factors can affect the degree of leaching and thus influence the success of this type of treatment. Sherburne *et al.* (276) used C^{14}-labelled monuron in a study of leaching and were able to show

how the distribution at different depths changed with different initial soil moisture, amount of simulated rainfall, and soil type. To some extent herbicides can be formulated to reduce leaching and the consequent risk of crop damage. Esters of 2,4-D, for instance, are leached less than the acid or the sodium or amine salts and are safer for the pre-emergence treatment of maize (7). The inclusion of large amounts of certain electrolytes or small quantities of water-insoluble organic acids in formulations of NPA (N-(1-naphthyl)phthalamic acid) has been shown by Smith *et al.* (282) to reduce the mobility of this herbicide in soil.

The resistance of herbicides to downward movement in the soil may be governed by their adsorption on to soil constituents. Warren (326) has indicated the wide variation in adsorption between compounds, and graphs showing the influence of soil type on the adsorption of monuron and diuron (N-(3,4-dichlorophenyl)-N′,N′dimethylurea) appear in a review by Abel (1). Sund (295) found a marked correlation between the loss of ATA by adsorption and the base exchange capacity of different soils; the toxicity to tomato seedlings paralleled the amount of the herbicide that was recovered by water extraction of the soil. On the other hand, although CDAA (2-chloro-N,N-diallylacetamide) and CDEC (2-chloroallyl-N,N-diethyldithiocarbamate) may be adsorbed on clay colloids and organic matter they are still available and toxic to germinating seeds [Slife (280)].

The adsorptive powers of certain materials can be used to protect crop seeds against damage from herbicides applied to the soil surface. Weaver (328) and Smith & Ennis (281) demonstrated that a layer of activated carbon positioned in the soil above the crop seed gave some protection against 2,4-D by intercepting its downward movement to the emerging roots. Ripper (255, 256) has developed this technique and constructed a field-scale machine which in one operation drills sugar beet seed, partially fills in the seed furrow, sprays a band of adsorbent over the seed, fills in and consolidates the soil above it, and finally sprays a herbicide on to the soil surface. The pre-emergence herbicide kills the weeds germinating in the soil above the adsorbent but the latter protects the seed of the crop.

Herbicides applied to the soil surface may cause toxic effects even though they do not reach the germinating seeds or roots. Davis (75), studying the use of DNBP as a pre-emergence treatment in cotton, found that although rain soon after sowing could leach toxic amounts into the seed zone, in practice there was a much greater risk of injury to the cotton after emergence due to vaporisation of the herbicide from the soil surface. Light rain during a period of high susceptibility five to eight days after emergence increased this risk as there was greater volatilisation from a moist than from a dry surface. An application of lime reduced the hazard.

Herbicides may undergo changes in the soil which influence their effectiveness. The best known example is the conversion by soil microorganisms of the inactive 2,4-dichlorophenoxyethyl sulphate to 2,4-D. Investigations

on the importance of various soil factors that influence this conversion have been continued by Isleib (149). A somewhat similar example is the conversion of the γ-phenoxybutyric herbicides to the active substituted phenoxyacetic acids, caused by the enzyme system of some, but not all, plants (see p. 324). Webley *et al.* (329) have shown that a soil micro-organism can also effect this conversion. Thus the phenoxyacetic acid may become available for uptake by the roots after an application of a γ-phenoxybutyric herbicide to the soil. The decomposition of herbicides in the soil may result in the formation of intermediates which are themselves phytotoxic; there are indications that such products occur in the bacterial breakdown of 2,4-D [Audus & Symonds (18)]. In contrast to this the results of Riepma Kzn (254) indicate that stimulatory products are formed during the breakdown of DNC in soil.

Herbicides do not persist indefinitely in the soil. They may cease to be available to roots because of (*a*) leaching, (*b*) volatilisation, (*c*) chemical breakdown and (*d*) microbiological decomposition.

Few herbicides are leached sufficiently for them to be moved below the reach of plant roots. Some can be lost by volatilisation: CIPC (*iso*propyl-N-(3-chlorophenyl)carbamate), for instance, may disappear in this way. Its rate of volatilisation is dependent on soil type and is influenced by changes in formulation [Loeffler (186)].

Most herbicides decompose in the soil and this is the commonest cause of their disappearance. Several instances of chemical breakdown have been described. Hannah (122) has outlined the steps by which chloroacetamides and dithiocarbamates could be hydrolysed in soil and Kutschinski (176) has indicated the way in which dalapon (sodium) may be decomposed and volatilised from soil. Photochemical degradation of 2,4-D can occur under ultraviolet light [Bell (28)], and there is also evidence for the photodecomposition of monuron [Hill *et al.* (140); Birk (31)]. Such photodecomposition can be of practical importance only where little rain occurs and the herbicide remains on the soil surface.

Microbiological decomposition seems to be a major cause of the disappearance of herbicidal activity from soils. In recent years a large number of papers have been concerned with this topic and only a few can be mentioned here. Herbicides and microorganisms can interact on each other in two ways. The herbicide may interfere with some part of the microbial activity of the soil, or a component of the microflora may attack and decompose the herbicide. Adverse effects on the microflora seem to be exceptional. Workers in Louisiana have found that 2,4-D reduces microbial activity only at concentrations far above those which could result from normal rates of application in the field (60, 155, 156, 157, 192, 193). The same would appear to hold for MCPA (20, 21), MH (113), and DNC (79). However, there are exceptions. Otten *et al.* (229) found that polyborchlorate markedly affected nitrification, and Quastel & Scholefield (239)

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reported that monuron was a powerful inhibitor of soil nitrification.

The breakdown of herbicides by microorganisms seems to be much more universal and effective. A variety of species are capable of decomposing chlorinated phenoxyacetic acids (18, 153, 257, 285, 288, 289, 290, 324). The pathway of breakdown has attracted considerable attention, but different workers do not agree on the nature of the intermediates that are involved [Steenson & Walker (290); Evans *et al.* (87, 88)]; different microorganisms may well attack the molecule in different ways. There is evidence of the microbial decomposition of a variety of other herbicides, including MH (178), dalapon (302), DNC (152) and substituted ureas (140, 275). Many of these papers contain observations on factors such as soil moisture and temperature, which influence microbial activity and in consequence the persistence of the herbicide. In some instances the rate of disappearance has been studied in detail. Hill *et al.* (140) followed the breakdown of methyl-labelled monuron by the evolution of $C^{14}O_2$ from the treated soil and obtained a decomposition rate of 10 per cent in 90 days.

Root uptake.—Although much research has been directed to the study of the entry of inorganic ions into roots, surprisingly little attention has been paid to the entry of organic molecules. There is a requirement for more information on the manner of entry of herbicides into roots.

Blackman (33) has given a preliminary report of experiments on the time course of uptake of C^{14}-labelled 2,4-D from solution by the root systems of intact seedlings. With *Hordeum vulgare,* a species resistant to 2,4-D, uptake continues steadily for 32 hours. With *Helianthus annuus,* a susceptible species, uptake is at first rapid, but later much of the 2,4-D is transferred back into the solution. With *Sorghum vulgare,* which is intermediate in susceptibility, uptake at first follows the same pattern as for *H. annuus,* but within a short time the loss of 2,4-D is succeeded by a slow recovery of the uptake mechanism. Blackman suggests that these differences in the behaviour of the three species are relevant to the elucidation of the basic differences between susceptible and resistant plants. Earlier work with the aquatic plants *Lemna minor* and *Salvinia natans,* where the uptake is mainly through the fronds, had shown that at high concentrations (16 to 48 p.p.m.) uptake and loss of 2,4-D by these species followed a sequence similar to that described for *H. annuus,* and both processes were found to be temperature dependent [Blackman (32)].

Movement within Plants

Work published since van Overbeek's recent review (317) has not fundamentally altered the well established picture of two main mechanisms by which herbicides may move: with the transpiration stream in the xylem, or with assimilates in the phloem. A comprehensive review of the translocation of herbicides will therefore not be attempted here; it may be useful, however, to call attention to certain pitfalls in current methods of research

on the subject, before briefly referring to some more recent investigations.

Methods of investigation.—The methods used to determine the distribution of herbicides in treated plants fall into three groups: (*a*) toxic, formative or nastic effects are observed at various distances from the site of application of the herbicide; (*b*) extracts of tissues are suitably purified and the concentration of herbicide in them is determined by bioassay (chemical methods are rarely sufficiently sensitive); or (*c*) herbicides labelled with a radioactive isotope are applied, and the distribution of radioactivity in the plant is determined by autoradiography or by electronic counting. All these methods are useful but each has limitations which are sometimes insufficiently recognised.

The picture given by observations of the first type may be incomplete because not all regions of the treated plant are capable of showing a visible response to the herbicide. The various bioassay and tracer methods can in principle be applied to any part of the plant, but caution is necessary in relating the results to weedkilling practices because from analytical data alone it may not be possible to tell whether the amounts of herbicide detected are at all similar to those necessary to affect the plant in the desired way. Unless the assay data are correlated with the effects on the treated plants one cannot be sure that the movements observed resemble those of effective amounts of the herbicide.

When a radioactive herbicide has been used, the distribution of radioactivity in the plant shows only the distribution of the labelled element and not necessarily that of the molecule in which it was originally incorporated. Many studies have demonstrated that herbicides undergo chemical transformations in plants [e.g. Fang & Butts (89); Fang *et al.* (91); Hay & Thimann (131); Holley (142); Jaworski *et al.* (151); Rogers (259); Weintraub *et al.* (339)]. Caution is therefore desirable before observations, whether by counting or by autoradiography, on the distribution of radioactivity are interpreted as showing the distribution of the herbicide, unless corroboration is available from the analysis of extracts.

In autoradiography the method of drying the plants has been shown by Pallas & Crafts (230) to affect drastically the distribution of radioactivity. They have established that post-mortem movements are minimised by rapid freezing with solid carbon dioxide, followed by drying the frozen material in a vacuum. When C^{14} is the isotope used, it seems possible that variations in structure and in density between different plant organs may be sufficient to affect the self-absorption of the low energy radiation and so to modify the picture obtained.

No conclusion about the route of movement of a herbicide through a translocating organ should be drawn merely from the distribution of the substance within it. The tissue with the highest concentration may be not the channel of movement but rather a site of accumulation and immobilisation. If one finds that at a given moment 90 per cent of the cars in a city

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street are distributed along the curbs, while only 10 per cent are in the central carriageway, one does not infer that the paths of movement lie along the edges of the road.

Finally, whatever method is used to determine the distribution of the herbicide, it must be remembered that the unaltered compound may not be the physiologically active substance [Weintraub *et al.* (337)].

Entry and immobilisation.—Translocation may be limited by the rate of entry into the plant, or by the immobilisation of the herbicide near the site of entry. Herbicides may move farther after application to a cut surface than after application to the intact plant, as reported by Bragg (44) for 2,4-D (amine) in *Asclepias syriaca,* by Hay (129) for 2,4,5-T (ester) in *Dichrostachys nutans,* and by Baldwin *et al.* (22) for CIPC in *Avena sativa* and *Zea mays.* Crafts *et al.* (70) considered that accumulation of 2,4-D by living cells may restrict its movement relative to that of other herbicides. Rice & Rohrbaugh (251) applied 2,4-D to the leaves of de-starched bean plants which were then kept for a period at various temperatures in darkness. Export of 2,4-D after transference to light was less in the plants which had been at high temperatures, suggesting that exhaustion of reserves or some other metabolic process was immobilising the herbicide. Hull (146) compared various emulsifiers when applying 2,4,5-T in oil-water emulsions to a few leaves of *Prosopis juliflora* seedlings and found that contact injury was inversely correlated both with epinasty near the stem apex and with damage to leaves remote from those treated. Like Leonard & Crafts (179) and earlier workers he concluded that local injury by a high concentration of herbicide in tissues near the site of application could reduce translocation. Hay (129, 130) concluded from his experiments on *Dichrostachys* that phytotoxic amounts of 2,4-D and 2,4,5-T were prevented from moving down the plant through living tissues because the herbicides disrupted the requisite transport mechanism.

Condition of the plant.—The physiological condition of the plant at the time of treatment may decisively affect both translocation and the action of the translocated herbicide. Leonard & Crafts (179), studying various species of brushwood, concluded that "for successful results, at least four physiological factors must be favourable: (*a*) the herbicide must be absorbed; (*b*) photosynthesis must provide assimilates for movement through the phloem; (*c*) translocation from active, photosynthesising leaves to the roots must be going on and (*d*) root activity must involve meristematic activity and growth, not just storage." Crafts (68) found that even when conditions allowed translocation of the herbicide from leaves to roots, treatment with 2,4-D might fail to kill *Convolvulus arvensis.* This occurred when lack of moisture limited the growth and hence the susceptibility of lateral roots, although deeper roots with a better water supply were killed by the same treatment. Fisher *et al.* (96) placed 2,4,5-T on leaflets of *Prosopis juliflora* and measured the distance below the point

of application to which the woody tissues were killed. Tests at 10 day intervals showed the greatest translocation 50 to 90 days after the first leaves appeared in spring. Concurrent observations of root kill and carbohydrate concentration in roots and stem gave results consistent with the view that for 2,4,5-T treatments to be effective there must be "active synthesis and translocation of carbohydrates by a high percentage of the leaves of a normal to heavy foliage cover." Yamaguchi & Crafts (353) have briefly reported that translocation of 2,4-D in *Zebrina pendula* was greatly affected by the rate of growth of the plant. Rohrbaugh & Rice (260, 261) have shown that phosphorus and potassium deficiency in tomato plants reduced the export of 2,4-D from treated leaves.

Xylem transport.—Turning to mechanisms of translocation, reports of acropetal movement in the xylem include those by Hay (130) for 2,4-D; Carvell (56) for ammonium sulphamate; Blanchard (38) for TCA (sodium); and Haun & Peterson (127), Minshall (206), Muzik *et al.* (216) and Normand (220) for monuron. Probably any chemical which can enter roots may be carried in the transpiration stream, but differences between compounds and between species deserve investigation.

Phloem transport.—No full discussion of movement in the phloem is possible without reference to fundamental theories of the mechanism of translocation which cannot be dealt with here; some relevant investigations will however be briefly noted. Jaworski *et al.* (151) found that the export of 2,4-D from the leaves of etiolated bean seedlings in the dark was promoted by the application of sucrose and glucose but not by a variety of other substances. Hay & Thimann (132) used bioassays of tissue extracts to follow the movement of 2,4-D applied in aqueous solution to a cut flap of the primary leaf of *Phaseolus* seedlings. They stated that the transport, which was accompanied by extensive chemical breakdown, ceased at about the middle of the hypocotyl, no 2,4-D entering the roots at any time. Export from the leaves was inhibited by ringing and by the application of excessive doses. Evidence was quoted to support the view that a metabolic component is involved in 2,4-D transport. The same conclusion was drawn by Barrier & Loomis (24) from experiments showing the high temperature coefficient of 2,4-D translocation in soyabean seedlings. Crafts (67) has described extensive researches on the mechanism of translocation as studied with labelled 2,4-D, giving a detailed picture of movements of the herbicide accompanying and determined by the movements of assimilates in the plant. Interesting comparisons between the translocation of variously substituted phenoxyacetic acids were mentioned by Weintraub *et al.* (337); in different plants the relative mobilities of the substances were not the same, and such differences may well affect the selectivity of the compounds when used as herbicides.

Information on the translocation of herbicides other than growth regulators is as yet scanty, and in particular more detailed evidence on the

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relation between their movement and that of photosynthates would be welcome. Clor & Crafts (59) briefly reported experiments showing that in cotton plants 2,4-D, ATA and urea moved at the same rate and with the photosynthate stream. In another preliminary report Crafts *et al.* (70) stated that MH was freely moved throughout *Tradescantia* plants; ATA was also readily mobile but to a lesser degree; 2,4-D and 2,4,5-T were accumulated by living cells and were less thoroughly distributed. Monuron moved only acropetally in the treated leaf and was not transported downward by the phloem; Fang *et al.* (91) found likewise that although monuron rapidly spread from the point of application throughout treated leaves of *Phaseolus vulgaris,* little was exported to the rest of the plant. Santelmann & Willard (266) concluded that dalapon was probably transported with photosynthates in *Agropyron repens,* but this was not the only means of translocation. Rogers (259) described the high mobility of ATA in plants, and Bondarenko & Willard (39) briefly reported effects of light on its movement from leaves to roots of *Cirsium arvense.*

Polar transport.—It has been stated (317) that synthetic auxins are not moved by the polar transport system which moves IAA through excised segments of plant parts. However, McCready (198) has found that phenoxyacetic acids were transported through short segments excised from young petioles of *Phaseolus vulgaris* by a mechanism which, although slower, in many ways resembles the polar transport of natural auxin. The movement was strongly unidirectional; it proceeded against a concentration gradient; it showed no dependence on carbohydrate movement; and it was highly sensitive to low temperatures, to metabolic inhibitors, and notably to triiodobenzoic acid [Niedergang-Kamien & Skoog (218) showed the latter to be a powerful inhibitor of the polar transport of natural auxin]. At low concentrations, comparable segments transported in unit time about 10 times as much 2,4,5-T as 2,4-D, while the movement of 2,6-dichlorophenoxyacetic acid was about one-tenth that of 2,4-D. The movement of 2-chlorophenoxyacetic acid and unsubstituted phenoxyacetic acid was less than one-hundredth that of 2,4-D. If some such mechanism moves externally applied herbicides into the pholem, its selectivity may be significant in determining whether or not a particular substance is translocated.

Species differences.—Differences in plant susceptibility to a given herbicide are the basis of selective weedkilling, and several investigators have sought the explanation in differences between the ability of susceptible and resistant species to translocate the herbicide. In general, dicotyledons are more susceptible than grasses to the phenoxyacetics, and it has been suggested that the intercalary meristem at the base of monocotyledonous leaves may reduce the export of herbicides from foliar applications [Fang & Butts (89)]. In a preliminary report Weintraub *et al.* (337) referred to experiments showing that in three dicotyledonous species the

export of 2,4-D from treated leaves was five to seventy times as great as in six species of Gramineae. Williams (346) compared the translocation of 2,4-D applied to the leaves of three annual dicotyledons—*Xanthium commune* (extremely sensitive to 2,4-D), *Datura stramonium* (intermediate) and *Sicyos angulatus* (resistant). He concluded that absorption occurred as rapidly in the susceptible as in the resistant plants, and that translocation took place in greater amounts in the latter. But the significance of such quantitative comparisons between species differing extensively in morphology and perhaps in metabolism is difficult to assess. Weintraub *et al.* (337) have briefly described some interesting differences in the translocation of radioactivity from labelled 2,4-D applied to the leaves of susceptible and resistant varieties of a single species, *Zea mays*. The total amounts of C^{14} which moved in 24 hours from the sites of application were virtually the same, but in the susceptible variety about half the mobile C^{14} had been exported from the blade and about one-third had moved out of the leaf altogether. In the resistant variety only one-fifth had left the blade and only one-sixteenth had been exported from the leaf. This is one of the rare experiments on whole plants in which a difference in translocation can be clearly distinguished from a difference in absorption. After similar treatments the specific activity of the C^{14} in parts other than the treated leaves of three susceptible varieties averaged twice that in three resistant varieties. This promising line of investigation strongly suggests that variations in translocation affect resistance and susceptibility.

Auxin Herbicides

The term auxin herbicides is used in this review for all herbicides that increase cell elongation in shoot tissue and have a physiological action resembling that of IAA (312).

Phenoxyacetic acids.—The importance and versatility of the three original—and still the most useful—auxin herbicides, 2,4-D, MCPA, and 2,4,5-T, have been adequately dealt with in previous reviews (37, 65, 219, 316). The more recent literature continues to be largely concerned with one or other of these three compounds, partly because there is still much to be learned of their capabilities as herbicides and partly because they have come to be regarded as standards in the evaluation of auxin herbicides and in physiological and biochemical studies concerning the mode of action of this type of chemical. Agronomic studies in many parts of the world have demonstrated the practicability of their use for the control of weeds in an increasing number of crops. New developments in formulation have also added new situations in which they can be used by increasing their efficiency and reducing the dangers of their drift on to susceptible crops.

With this clearer understanding of the capabilities as well as the limitations of 2,4-D, MCPA, and 2,4,5-T and their many formulations, have

come reports of the reappraisal of other members of the substituted phenoxyalkylcarboxylic acids and their derivatives [Burström *et al.* (55); Leafe (177); Lush (189); Shaw & Gentner (273); Wain (322)]. Several of these chemicals possess desirable herbicidal characteristics and in recent years the following have become available commercially:

γ-(2,4-dichlorophenoxy)butyric acid, [4-(2,4-DB)]
γ-(4-chloro-2-methylphenoxy)butyric acid, [4-(MCPB)]
α-(2,4,5-trichlorophenoxy)propionic acid, [2-(2,4,5-TP)]
α-(4-chloro-2-methylphenoxy)propionic acid, [2-(MCPP)]
2,3,6-trichlorobenzoic acid, [2,3,6-TBA]

γ-Phenoxybutyric acids.—The fundamental studies that led to the development of the phenoxybutyric acid herbicides, 4-(2,4-DB) and 4-(MCPB) have been described in a series of papers by Wain and his colleagues (92, 321, 323) and were considered in the review by Muir & Hansch (214). Experiments on whole plants have amply confirmed the fact that species differ markedly in their relative susceptibility to the acetic and γ-butyric homologues of 2,4-dichloro-, 4-chloro-2-methyl-, and 2,4,5-trichlorophenoxy acids [Fryer & Chancellor (103); Shaw & Gentner (273)]. Thus, merely by adding two methylene groups to the side chain of a herbicidal phenoxyacetic acid it is possible to change its relative toxicity to different species. This change in specificity is believed to be associated with the ability of the plant to convert the inactive butyric compound into the active phenoxyacetic acid.

Field experiments in many parts of the world have shown that 4-(2,4-DB) and 4-(MCPB) both possess a degree of selectivity to crops and weeds which enables them to be used in situations where the corresponding acetic derivatives are too toxic to the crop. In general the butyric herbicides are comparatively nontoxic to legumes, and it is in such crops as clover, peas, and lucerne that they are most successful. British results have been summarized by Fryer & Evans (104).

The γ-(2,4,5-trichlorophenoxy)butyric acid [4-(2,4,5-TB)] is relatively nontoxic to most species but does kill the bindweeds, *Calystegia sepium* and *Convolvulus arvensis* [Luckwill & Campbell (188)]. This specificity may prove of value in a few crops and already 4-(2,4,5-TB) is being advocated for the control of bindweed in blackcurrants. There are indications that several other perennial weeds are just as susceptible to 4-(2,4-DB) and 4-(MCPB) as they are to the corresponding phenoxyacetic acids, 2,4-D and MCPA, and it has been suggested that this is the result of a slow internal conversion of the phenoxybutyric acid to the acetic acid homologue which results in a lower dose spread over a longer time. If this were true, then the phenoxybutyric herbicides might be expected to be slower acting. Holly (144) found no evidence for this with a number of species, but in two experiments on *Sinapis alba* the ratio of the amounts

of 4-(MCPB) to MCPA required to produce the same effect decreased as the time interval between treatment and the measurement of the response was increased. In practice it has also been observed that some species, particularly *Sinapis arvensis,* become resistant to 4-(MCPB) earlier than to MCPA.

Much emphasis has been given to the theory that the selective action of the γ-phenoxybutyric herbicides is due to the presence or absence of specific β-oxidation systems in different species. Very little is known concerning the location and behaviour of these enzymes in the living plant, and it seems possible that some of the differences between the phytoxicity of the γ-phenoxybutyric acids and their acetic homologues could equally be explained by differences in uptake, penetration, and translocation.

Phenoxypropionic acids.—Several phenoxypropionic acids have been tested as selective herbicides [Shaw (272)]. Only two, 2-(2,4,5-TP) and 2-MCPP are used commercially; 2-(2,4,5-TP) is as toxic as 2,4,5-T to several tree species [Darrow (74)] but less toxic to some crops, particularly cotton. In consequence it is employed in preference to 2,4,5-T for the aerial treatment of susceptible trees (e.g. *Quercus marylandica* and *Q. stellata*) growing in the vicinity of cotton. The interesting selective phytotoxic properties of 2-MCPP have only recently been discovered [Leafe (177); Lush (189)]. This chemical kills several weeds that are resistant to 2,4-D and MCPA and is less toxic than these herbicides to cereals. It has been used extensively in Great Britain during 1956-57 particularly for the control of *Galium aparine* in cereals.

Benzoic acids.—Formulations of 2,3,6-TBA, 2,3,5,6-tetrachlorobenzoic acid, and mixtures of the two, with and without additional isomers, are being tested as pre- and post-emergence herbicides. Most of the field experiments have been carried out with mixed isomers. It is, therefore, not possible to obtaine a true assessment of their relative toxicity, although it is generally assumed that 2,3,6-TBA is the most herbicidal form. The relationship between chemical structure and growth regulating activity of the substituted benzoics has been reviewed by Muir & Hansch (214). The formative effects caused by 2,3,6-TBA are quite distinct from those induced by 2,4-D. In monocotyledonous species it affects nodal meristems often causing a weakness that results in lodging, while in dicotyledons, apical meristems are often completely inhibited and although the plant is incapable of growth it does not die. In this respect it is somewhat similar to 2-(MCPP). 2,3,6-TBA is very persistent in soils [Crafts (69)].

Other phytotoxic auxins and related compounds.—Since the last review (65) there have been many reports of the phytotoxicity of growth substances of the auxin type. The most interesting of these deal with phenoxythioacetic acids [Burström *et al.* (55)]; 4-fluoro- and 4-bromophenoxyacetic acids [Ready & Minarik (242)]; 2,6-phenoxyalkylcarboxylic acids [Osborne *et al.* (228); Toothill *et al.* (308)]; amino acid derivatives of

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MCPA, 4-chlorophenoxyacetic acid and 2-(2,4-dichlorophenoxy)propionic acid [Krewson *et al.* (172, 173, 174)]; chlorinated aryloxymethylphosphonous and phosphonic acids [Greenham (112)]; 1,3-benzodioxole-2-carboxylic acids [Zimmerman *et al.* (355)]; and analogues of 2-mercaptobenzimidazole [Rebstock *et al.* (243, 244)].

Effects on Plants

The literature concerning the anatomical and physiological changes brought about by auxins is extensive. It ranges from papers dealing with the evaluation of auxins as herbicides to those designed to study their action at cell level. No attempt has been made in this review to cover the whole field and we have omitted all references to anatomical and cytological changes.

The abnormal processes that result from the introduction of an incompatible chemical are likely to be just as complicated as those of normal growth. An effect may be observed in a matter of minutes, as with the streaming of protoplasm, or be delayed several months as when grain is analysed to determine the effect of a herbicide applied to the plant when it had only two or three leaves. Attempts to relate cause and effect, when the time interval between the application of the herbicide and the measurement of the response extends over the greater part of the life cycle of the plant, are fraught with difficulties and can easily confuse rather than clarify an understanding of the mechanism of action of the herbicide.

Protoplasmic streaming.—Kelso & Turner (169), using the staminal hair cells of *Tradescantia,* have measured the effects of IAA and other auxins on protoplasmic streaming; Kefford & Kelso (168) claim that the technique is a useful method for the assay of growth regulating activity. The difficulties of interpreting changes in protoplasmic streaming, which are influenced by many factors and may merely reflect changes in viscosity, have been pointed out by Currier (71). Nevertheless, the results obtained by Kelso & Turner do show extremely interesting differences in the responses to the different growth regulating substances, particularly IAA and 2,4-D. At concentrations below 100 p.p.m. the stimulatory and inhibitory effects of IAA were temporary. In contrast the stimulatory effect of 2,4-D was less, took longer to reach a maximum, and was permanent. It resembled the effects obtained with IAA in the presence of malic acid and sugars.

Stomata.—2,4-D when applied to soil [Ferri & Rachid (95)] or to leaves [Bradbury & Ennis (41)] causes the stomata of many species to close. The effect is proportional to the dose applied to the leaves, over the range from 10 to 1000 p.p.m. (41), and seems to be the result of a direct action on the leaves and stomatal cells rather than an indirect effect on the water-absorbing mechanism of the roots. This stomatal closure is associated with a decrease in the phosphorylase and amylase of the stomatal cells [Maciejewska-Potapczyk (190)].

Photosynthesis.—The earlier work of Freeland (100) showing that 2,4-D inhibits CO_2 assimilation has been confirmed by Akers & Fang (6) by growing *Phaseolus vulgaris* in a closed system containing $C^{14}O_2$. A more drastic technique of leaf infiltration has also shown that the inhibition of photosynthesis is proportional to the concentration of undissociated 2,4-D in the external solutions [Wedding *et al.* (332)]. Blackman & Robertson-Cuninghame (36), employing techniques of growth analysis, demonstrated that reductions in the growth of *Helianthus annuus* caused by 2,4-D were due to a decrease in the " leaf area ratio" rather than a diminution in the net assimilation rate. The same authors made comparable studies on the aquatic plant *Lemna minor* (35).

Uptake.—2,4-D at 3×10^{-4} *M* and above inhibited the metabolic component of IAA uptake by pea epicotyls and carrot discs [Reinhold (248)]. This apparently noncompetitive inhibition of IAA uptake could explain some of the growth regulating effects of 2,4-D. It certainly has to be taken into consideration in studies on antagonism where more than one growth regulator is present in the ambient solution or spray liquid used for applying the treatment.

It is generally assumed that auxin herbicides cause an inhibition of mineral uptake, and most of the papers on this subject are concerned with the varying magnitude of this inhibition for individual elements by different species [Tomizawa (305); Wildon *et al.* (344)]. Blackman (32) has emphasized that the changes in mineral content may be due to a direct effect of 2,4-D on absorption or an indirect effect resulting from the retardation of growth. The immediate effect of an auxin on uptake may, however, be stimulatory. Cooke (63) has shown that bean plants sprayed with 2,4-D absorbed much higher quantities of $^{42}K^{+}$ than the control plants during the first 8 to 12 hours, but that this was followed after 24 hours by a marked decrease. The uptake of $^{36}Cl^{-}$, $^{45}Ca^{++}$ and $S^{35}O_4^{=}$ changed similarly.

Protein metabolism.—There are numerous records of the effects of 2,4-D and other similar herbicides on the nitrogenous compounds of treated plants [Crafts (65)]. Most experiments relate to the gross changes in plant composition brought about by herbicidal doses applied many weeks before the plants were sampled for analysis. They are, in consequence, often of little value in the interpretation of the primary effects of the herbicide.

Fults & Payne and their colleagues (184, 354) have continued their detailed studies of the effects of foliar applications of 2,4-D and other herbicides on the free amino acid and protein content of sugar beet roots and potato tubers at harvest and after storage. Similar work on the foliage of these crops and of beans soon after treatment, has led them to suggest that the observed differences in the responses of different species may be intimately connected with the selective action of 2,4-D (105). Freiberg & Clark (101) added 2,4-D to a nutrient culture, which was devoid of

nitrogen during and after treatment, and analysed soyabean plants grown therein one, three, and five days later. Their results indicate that 2,4-D caused hydrolysed protein to move from the leaves into the stem and root where it was resynthesised.

There is accumulating evidence for links between auxin action and amino acid metabolism: Reinhold & Powell (249) have shown that IAA increased the uptake of glutamic acid and other amino acids by sunflower sections, while Andreae & Good (11) and Good *et al.* (109) have isolated auxin aspartic acid peptides from various tissues. 2,4-D also affects the metabolism of amino acids, probably causing an increase in their rate of oxidation or catabolism [Akers & Fang (6)].

Phosphorus metabolism.—The close association between auxin action and respiration is well established, and although it is not yet clear whether auxin acts directly on the respiratory mechanism or via its effect on growth, the phosphate metabolism of the organism must be involved. Van Overbeek (316) has suggested that the primary action of auxin is linked with this metabolism, and there are many papers concerning the effect of 2,4-D on the inorganic and organic phosphorus content of plants.

The inorganic fraction usually increases after treatment [Loustalot *et al.* (187); Rakitin *et al.* (241)]. Tomizawa (305) obtained evidence to suggest that this was probably due to an inhibition of the incorporation of the inorganic phosphorus into intermediates, such as hexosediphosphate; however, Fang & Butts (90) found an increase in the amount of P^{32} incorporated into this compound. In soyabean mitochondria 2,4-D at $5 \times 10^{-4}M$ inhibits phosphorylation [Switzer (298)].

When whole plants are treated, the phosphorus content decreases in the leaves [Rebstock *et al.* (246); Tomizawa (305); Fang & Butts (90)] and sometimes increases in the stems. This could be the result of movement from the leaves (246, 305) or of a reduction of movement towards them (90).

Respiration.—It has been recognised for many years that auxin induced stimulation of growth is usually accompanied by an enhanced rate of respiration, and there are many records of increases in respiration as a result of 2,4-D treatment [Henderson *et al.* (137); Kandler & Neumair (165); Levari (182); Linden (183); Wedding *et al.* (332); Yamada & Murata (352)]. Bonner (40) and French & Beevers (102) have put forward the view that the increased rate of respiration is not due to a direct action of auxins on the oxidative systems of the cell, but is the indirect result of the stimulation by auxin of some energy-requiring process. Thus adenosine triphosphate consumption will be increased under the influence of auxin, bringing about a regeneration of the phosphate acceptor, the availability of which is regarded as limiting the respiratory rate. However, Switzer (298) has shown that 2,4-D inhibits both oxidation and phosphorylation in soyabean mitochondria, and Remmert *et al.* (250), using mito-

chondria from cabbage, have obtained results which indicate that the 2,4-D inhibition may depend largely on an effect on the transport of electrons between reduced diphosphopyridine nucleotide and oxygen.

Humphreys & Dugger (147) observed that in etiolated pea seedlings the increased rate of respiration took place without a change in R.Q. They have measured the relative amounts of glucose catabolised via the pentose phosphate pathway and the classical glycolytic pathway in short period experiments with root tips of pea, maize, and oat seedlings previously treated with 2,4-D and IAA. In all seedlings tested, 2,4-D caused an increase in the amount of glucose catabolised via the pentose phosphate pathway, but IAA did not influence glucose metabolism in the same manner. These results are consistent with the conclusion drawn by Smith (284) in his review on respiratory changes in relation to the toxicity of growth regulators, namely that the fundamental effect is probably a change in type of respiration, which may be accompanied by either an increase or an inhibition of total respiration.

Enzymes.—2,4-D is the compound most frequently used in experiments designed to measure the effects of synthetic auxins on enzyme systems and Wort (350) has compiled a table summarising the findings up to 1954. The results of later work are summarised in Table I. This table also records, where possible, comparative data for IAA. In most instances the herbicide was applied to the plant and the activity of the enzyme assessed subsequently. Such a technique may measure responses that have no direct bearing on the mode of action of the herbicide. Nevertheless, the results obtained are valuable in characterising the changes induced and may show up important differences either between compounds or between the responses of resistant and susceptible species [lipase, Weintraub (335); catalase, Tsitovich (309); amylase, Kasperlik (166)].

There are several records of 2,4-D having caused a slight increase in enzyme activity, but there are no reports of major differences between the action of 2,4-D and IAA. It seems unlikely that any of the recorded effects on enzymes could be responsible for the growth regulating action of auxins. The Italian school, however, claim that an inhibition of ascorbic acid oxidase by auxins reduces the level of dehydroascorbic acid, which is inhibitory to several respiratory enzymes [Marrè & Arrigoni (196); Tonzig & Marrè (307)]. It must be remembered, however, that Newcomb (217) obtained a marked increase in ascorbic acid oxidase activity when he grew tobacco pith sections in the presence of IAA.

The results that Osborne (227) has obtained with peroxidase and pectin methylesterase are pertinent examples of the necessity for care in the interpretation of responses obtained from the application of auxins to living tissue whose native enzyme status is changing with time. Osborne finds that peroxidase activity increases in the pulvinus and petiole of excised abscission zones from bean plants as these isolated sections proceed toward

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TABLE I

THE EFFECTS OF AUXIN HERBICIDES ON ENZYMES

Enzyme	Species	Method of treatment	Chemical and concentration	Effect	Reference	
Amylase	Rice	Spray on seedlings	2,4-D	Variable	Tomizawa & Koike	(306)
	Sweet potato	Spray	2,4-D	Nil	Tomizawa & Koike	(306)
	Several species	Application (leaf and root)	2,4-D	Varied with species	Kasperlik	(166)
	Pelargonium	Spray on leaves	2,4-D 0.1%	Inhibition	Maciejewska-Potapczyk	(190)
Ascorbic acid oxidase	Pea	Mitochondrial preparation	4-CPA $5\times10^{-4}M$	Inhibition	Tonzig & Marrè	(307)
	Pea	Purified extract	IAA $5\times10^{-4}M$	Inhibition	Marrè & Arrigoni	(196, 197)
Catalase	Wheat	Spray on leaves	2,4-D	Stimulation slight	Tsitovich	(309)
	Maize	Spray on leaves	2,4-D	Inhibition	Tsitovich	(309)
	Potato	Spray on leaves	2,4-D	Inhibition	Tsitovich	(309)
	Tomato	Spray on leaves	2,4-D	Inhibition	Tsitovich	(309)
	Jerusalem artichoke	Tissue	2,4-D $10^{-4}M$	Inhibition	Morel & Démétriades	(211)
			IAA $10^{-4}M$	Inhibition	Morel & Démétriades	(211)
	Rice	Spray (seedlings)	2,4-D	Stimulation slight followed by inhibition	Tomizawa & Koike	(306)
	Sweet potato	Spray	2,4-D			
Dehydrogenases	Rice	Seeds in culture	2,4-D	Varies with substrate	Tomizawa & Koike	(306)
IAA oxidase	Pea	Tissue	2,4-D $10^{-1}M$	Stimulation	Galston & Dalberg	(107)
	Bean (*Phaseolus*)	Excised abscission zones	2,4-D 5, 10 and 20 p.p.m.	Depresses the normal increase that occurs as tissue ages	Osborne	(227)
Invertase	Rice	Spray (seedlings)	2,4-D	Stimulation slight followed by inhibition	Tomizawa & Koike	(306)
	Sweet potato	Spray	2,4-D			

TABLE 1 (*continued*)

Enzyme	Species	Method of treatment	Chemical and concentration	Effect	Reference
Pectin methyl esterase	*Cladosporium herbarum*	With pectin substrate	2,4-D 2,4,5-T	Stimulation followed by inhibition	Waygood, Kapica & Mozie (327)
			IAA $10^{-5}M$	Stimulation	Waygood, Kapica & Mozie (327)
			IAA $10^{-3}M$	Inhibition	Waygood, Kapica & Mozie (327)
	Bean (*Phaseolus*)	Droplets on leaves Excised abscission zones	2,4-D, 2,4,5-T 2,4-D 10–40 p.p.m.	Retards the normal decrease that occurs as tissue ages	Osborne (227)
Peroxidase	Jerusalem artichoke	Tissue	2,4-D $10^{-6}M$	Stimulation slight	Morel & Démétriades (211)
			IAA $10^{-6}M$	Stimulation slight (higher than 2,4-D)	Morel & Démétriades (211)
	Bean (*Phaseolus*)	Excised abscission zones	2,4-D $5\times10^{-6}M$	Retards normal increase as explants age	Osborne (227)
Phosphatase	Rice	Spray (seedlings)	2,4-D	Stimulation	Tomizawa & Koike (306)
	Sweet potato	Spray	2,4-D	Stimulation	Tomizawa & Koike (306)
Glucose-6-phoaphate dehydrogenase	Pea	Homogenate	IAA $10^{-6}M$ 2,4-D $5\times10^{-7}M$ 4-CPA $5\times10^{-7}M$	Stimulation	Marrè & Arrigoni (195)
Polyphenol oxidase	Jerusalem artichoke	Tissue	2,4-D $10^{-6}M$	Stimulation	Morel & Démétriades (211)
Phosphorylase	*Pelargonium*	Spray (leaves)	2,4-D 0.1%	Inhibition	Maciejewska-Potapczyk (190)
	Tomato	Spray (flowers & leaves)	2,4-D 15 p.p.m.	Stimulation	Yakushkina (351)

maturity, and that 2,4-D concentrations which prevent abscission merely inhibit the natural rise in peroxidase activity that occurs as the tissue ages. The pectin methyl esterase activity, on the other hand, decreases in untreated tissue and doses of 2,4-D and 2,4,5-T that prevent abscission merely delay the natural decrease in enzyme activity. Thus the action of 2,4-D in these two instances is to maintain the level of the enzyme unchanged when it would normally be rising or falling.

Mechanism of Action

Until the mechanism of action of natural auxins is much better understood, it is unlikely that a clear picture of the toxic action of auxin herbicides will emerge, for it is the similarities and differences between the actions of the natural and the synthetic that are at the centre of this problem.

Much of the work on auxins has been concerned with the study of elongating cells of *Avena* coleoptiles and observations have been confined to the last 20 to 40 per cent increase in length of cells that during their lifetime have already grown approximately fiftyfold. Experiments dealing with this limited aspect of their action are unlikely to be of much help in explaining the general differences between herbicides and endogenous auxins which are concerned with the complex growth of the whole plant. More progress is likely to be made by the use of tissue culture techniques [Skoog & Miller (278); Steward & Shantz (291); Wetmore (342)] where studies of multiple growth responses can be made under controlled conditions of nutrient and growth factor supply. The suggestion that the quantitative interactions between auxins and kinins provide a mechanism for regulating all types of growth [Skoog & Miller (278)] indicates how the observed diversity of responses to a single auxin could be brought about.

The amounts of an auxin required to produce significant effects in different parts of a plant vary widely (303). For instance, the concentration of IAA required to inhibit stem growth is about one million times that required to inhibit root growth. Even within the same tissue different doses elicit different responses and sometimes it takes several weeks for the more slowly induced effects to appear [Ropp & Markley (262)]. It is likely, therefore, that the variety of growth regulating and toxic effects of auxin herbicides are the result of the many different concentrations of these substances that occur for different intervals of time in the various tissues and organs of the treated plant. These local concentrations will in turn be determined by the factors controlling the penetration, transport, and persistence of the compounds within the plant.

A common characteristic of all auxins is that at low concentrations they stimulate or accelerate plant metabolism and growth while at high concentrations they are usually inhibitory [Thimann (303)]. It is often assumed that the toxic action of herbicides is concerned solely with the inhibitory

rather than the stimulatory response. There is little justification for this viewpoint. It seems much more likely that the toxic effects of auxin herbicides, which often extend over many weeks in annual plants and longer in perennials, are made up of many different types of stimulatory as well as inhibitory effects.

Chemical structure and herbicidal or formative activity.—Much work has attempted to relate chemical structure to growth regulating activity in the hope of discovering more about the mode of action of auxins, but as Veldstra (320) has said "We have to admit that, despite all these efforts, our joint explorations have so far not yielded real basic information as to the mode of action." If this is true for the structural requirements associated with cell elongation in *Avena* coleoptiles, it is even more so with regard to the structural requirements of auxin herbicides. The selectivity of such herbicides is, in fact, concerned with the very variables that make it impossible to collate the range of biological responses that have been recorded for auxins of different chemical constitution [Muir & Hansch (214)]. In this connection it is interesting to recall that the discovery of the γ-phenoxybutyric acid herbicides arose from observations of the differences in biological responses to different members of the ω-phenoxyalkylcarboxylic acid series [Wain (321)].

Weintraub *et al.* (336) have indicated how some of the structural requirements for 'formative' activity differ from those for elongation growth. Burström and his colleagues at Lund have measured the effects of a large number of plant growth regulators on cell elongation, cell multiplication and toxicity (80, 85, 86, 161, 162, 164); compounds which stimulated cell elongation always caused a decrease in cell multiplication [Jönsson (163)]. 2,4-D and other phenoxycarboxylic acids promote the active growth of potato tuber tissue in culture [Steward & Shantz (291)] and the effects of ring substitution and side chain length on growth, cell number and cell size have been determined [Steward *et al.* (292)]. Such techniques, however, give little indication of the herbicidal properties of the chemicals. They are concerned with limited aspects of growth, whereas the reaction of the whole plant is determined largely by the ability of the chemical to enter, persist, and be translocated.

Theories of herbicidal action.—Many possible mechanisms of toxic action have been suggested. Leopold (181) lists the following: respiratory depletion, abnormal cellular proliferation, the production of toxic substances (lactones and amino acids), abnormal phosphatase activity, protein hydrolysis, and upset in potassium uptake and metabolism. Any of these effects could result in death, but in view of the extreme diversity of the responses that can be brought about by auxin herbicides it seems unlikely that any one could account for all the observed facts and that more general theories are required to explain the whole range of differences between endogenous and herbicidal auxins.

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It has been suggested that auxin herbicides might act by changing the level of endogenous auxin. In 1953, Weintraub (335) put forward a theory for the mode of action of 2,4-D which was based on the observation that this herbicide lowered the level of endogenous auxin. Normal growth and polarised divisions of meristematic cells are believed to be dependent on the level of endogenous auxin. If, therefore, this level is lowered below the minimum required, one might expect unpolarised division, widespread changes in metabolic and physiological processes, and ultimately death. Using the *Avena* assay for native auxin, Lockhart & Weintraub (185) have been able to show that the ether extractable auxin in the terminal shoots of bean plants treated with 2,4-D decreased within 24 hours after treatment and recovered in about seven days. Henderson has also obtained a reduction in the content of natural auxins in sunflower, pea, and bean, but not of oat (135, 136). In addition Weintraub (334) has prevented the 'formative' effect of low doses of 2,4-D by concurrent application of IAA. Audus & Thresh (19), on the other hand, have not been able to find any effect of 2,4-D on the levels of free endogenous IAA in broad bean, sunflower, and germinating pea when the plants were extracted one and seven days after treatment. The discrepancy between these two sets of results is possibly attributable to the fact that Audus & Thresh measured only IAA while Lockhart & Weintraub and Henderson estimated total endogenous auxin. Furthermore, the time intervals after treatment chosen for sampling by Audus & Thresh did not correspond with the times of maximum effect in Lockhart & Weintraub's experiments. Clearly, more experimental evidence based on the estimation of endogenous auxin extracted from different tissues at several times after treatment is required. Even so, there would be no proof that the effect of 2,4-D on endogenous auxin was intimately connected with the principal mode of action of this herbicide.

Other theories do not invoke the effect of the herbicide on the level of endogenous auxin and relate to the more direct effects of the toxic compound which may, however, take place at the same site of action as that of IAA. These toxic effects may be brought about by concentrations that cause a stimulatory response, or they may be the result of supra-optimal concentrations that are inhibitory to growth, or perhaps both.

(*a*) Stimulatory concentrations.—It seems probable that the growth-stimulatory actions of IAA and auxin herbicides are similar and take place at the same growth locus [Audus (16)]. Skoog (277) has long held the view, which is shared by Veldstra (320), that the basic metabolic acts of auxins are intimately connected with nucleic acid metabolism and there is now little doubt that the interactions of IAA and kinetin [6-(furfurylamino)] purine are associated with nucleic acid synthesis (278). The similarity in the structures of adenine, kinetin, and IAA has been discussed by Thimann (303). No one, however, seems to have recorded the fact,

brought to the attention of the authors by Brown (48), that the structural relationship between IAA and 2,4-D (or other phenoxyacetic acid herbicides) corresponds to a remarkable extent with that between the purines and pyrimidines and that in addition benzimidazole, which in chemical structure lies between IAA and purine, also possesses growth regulating properties which are intermediate between those of auxins and purines [Galston *et al.* (106)].

(*b*) Supra-optimal concentrations.—The toxic effects of supra-optimal concentrations may be the result of the same mechanisms that cause growth stimulation or they may be quite separate and physiologically distinct [Åberg (2, 3); Burström (53, 54); Hansen (123, 124)].

The theory that the inhibitory effects of supra-optimal concentrations of auxin are the result of competition for reactive sites [Foster *et al.* (97)] has become less tenable in view of the findings of Marinos (194). He has shown that a supra-optimal concentration of IAA, 2,4-D, or 2,4,5-T always caused an initial rapid rise in the elongation of *Avena* coleoptile sections and that this was later followed by a shrinkage associated with a loss of water and solid matter. Marinos suggests that the long term inhibitory action of high concentrations of auxins is due to secondary damage and shrinkage of the tissue and not to an immediate retardation of the growth processes. Bennet-Clark & Kefford (29) have put forward a similar explanation but also postulate that the retardation in elongation growth might be due to the accumulation of inhibitors. Thimann (303), however, seems to assume that the toxic effects of high IAA concentrations are a part of the true growth reaction, since he points out that studies concerned with protection against such inhibitory effects may be indicative of the site of action of auxins.

The situation in roots is more complex. There is still doubt that auxins cause a stimulation of the growth of intact roots [Åberg (4)], and it has been suggested that growth regulating substances should be divided into shoot auxins and root auxins [Hansen (123)]. The toxic effects of high concentrations of root auxins are apparently quite distinct from those caused by shoot auxins, are not relieved by them, and according to these Swedish workers are unconnected with the shoot auxin system.

The persistence of auxin herbicides in plants.—The ability of a herbicide to persist in an active form in plant tissues is likely to be of primary importance whatever the precise mechanism of action of the herbicide. A simple and attractive explanation of why IAA is useless as a herbicide, while many synthetic auxins are highly effective, is that plants contain a mechanism for the inactivation of their endogenous auxins when they are present in excess but have no such safety mechanism for the control of synthetic auxins.

Auxin control within the plant might be established by (*a*) destruction,

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(*b*) immobilisation due to the formation of association products, or (*c*) counteraction by inhibitors. Such detoxication mechanisms and the metabolism of auxins have been reviewed by Audus (16).

2,4-D persists in plants for much longer periods than IAA [Audus (16)]. Chemical separations of C^{14}-labelled 2,4-D have shown that it can persist in the dormant buds of two-year-old cherry trees for five months [Weintraub *et al.* (338)]. Bradley & Crane (43) studied in detail the effects on apricot seedlings of application of 2,4-D, 2,4,5-T, and 2-(2,4,5-TP) to the parent trees after flowering. They concluded that the active substances probably had persisted in the seed until germination seven months after treatment [see also Schuster (270)].

The suggestion that auxins may become attached to plasmatic surfaces and thereby inactive in growth regulation has been considered in reviews by Gordon (110) and Åberg (4). Brian (47) has studied the adsorption of MCPA onto films formed in a Langmuir trough from the expressed sap of different species and showed that the herbicide was adsorbed more strongly to the films formed from resistant species. Of particular interest in the understanding of herbicidal action is the work of Andreae, Good and van Ysselstein (10, 12, 109). IAA when fed to tissues of different species can subsequently be identified in the tissues as indoleacetyl-aspartic acid and indoleacetamide, the proportions depending on the species. The corresponding aspartic acid and amide derivatives of 2,4-D are found in much smaller quantities, if at all, and the chemical persists as the free acid. Indolebutyric acid and benzoic acid behave similarly to IAA. Andreae and Good (11) suggest that 2,4-D is phytotoxic because it persists in the tissue by resisting degradation and by failing to form conjugation products.

Normal growth of tissues or plants is often considered to be controlled by a balance between growth promoting and growth inhibiting systems [Steward & Shantz (291); Thimann (303)]. Although there are records of the chemical extraction of inhibitors, attempts to demonstrate the diffusion of inhibitory substances into the external solution have been unsuccessful [Bennet-Clark & Kefford (29); Osborne (226)]. This may be because the balance between growth promoters and growth inhibitors in the normal organism is so accurately maintained that it is difficult to upset the balance sufficiently in favour of the inhibitor. Situations have, however, been recorded where short period treatments with IAA have subsequently caused inhibition, suggesting that it is possible, under certain conditions, to upset the balance sufficiently for the presence of the inhibitor to be detected by growth measurements. Ball & Dyke (23), using whole seedlings of *Avena* which had previously been subjected to a short period of immersion in IAA, found that there was a stimulation of growth at first, but this subsequently fell below that of the control plants. Similar pretreatment with 2,4-D resulted in growth stimulation but no subsequent growth inhibition.

A more detailed examination of this problem has been undertaken by Osborne (226) who measured the growth responses of sections of etiolated pea internodes pretreated for short periods with IAA, 2,4-D, and 2,5-dichlorobenzoic acid. The growth of sections pretreated with suboptimal concentrations of IAA for a few hours and then transferred to water fell below that of the control sections kept in water all the time. In addition the sections did not subsequently respond fully to further applications of other growth regulators. When 2,4-D and 2,5-dichlorobenzoic acid were used for pretreatment instead of IAA, there was no subsequent inhibition of growth and the tissues responded fully to further applications of other growth regulators. The author suggests that treatment with IAA induces the formation of an inhibitory substance which accumulates within the tissue and dislocates the growth processes; the tissues are in consequence rendered less sensitive to further applications of growth regulators. There is no evidence for the formation of such an inhibitor following treatment with 2,4-D and 2,5-dichlorobenzoic acid.

In summary, IAA differs from 2,4-D in the following respects: (*a*) it is less persistent in plant tissues; (*b*) it is capable of forming inactive association products within the plant; (*c*) it may induce the formation of inhibitors which counterbalance its stimulatory action. It seems reasonable to suppose that whatever the mechanisms by which the auxin herbicides exert their effect, their potency will be enhanced by their ability to persist in plant tissues and to exert their growth regulating effects over extended periods of time. In addition the range of susceptibilities of different species may also be due in part to variations in their detoxication mechanisms.

Chloro- and Methyl-substituted Acetic Acids

The herbicides in the group are: mono-, di- and trichloroacetic acids, 2,2-dichloropropionic acid (dalapon), 2,2,3-trichloropropionic acid, and 2,3-dichloro*iso*butyric acid. As a group they tend to be more toxic to monocotyledonous than to dicotyledonous plants and are, in consequence, used mainly for the control of graminaceous weeds, either selectively in crops such as beet or lucerne (*Medicago sativa*) or in fallow land. Their phytotoxic effects are similar. All cause leaf chlorosis and abnormal growth responses typical of plant growth regulators. In addition to these systemic effects, the mono-, di-, and trichloroacetic acids have a pronounced contact toxicity which is associated with an inability to be translocated from leaves. They cause slight increases in the elongation growth of wheat coleoptiles at concentrations near toxic levels but have no activity in the pea curvature test. Fawcett *et al.* (93) suggest that their small effect on elongation may be due to changes in membrane permeability or cell wall structure as a result of sub-acute toxicity, and they should not be classified as true growth regulators. Against this must be set their ability to cause marked formative effects.

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TCA has been widely used as a herbicide since 1949 and was considered in the last three reviews (37, 65, 219). The monochloroacetic acid had until this year been considered almost solely as a desiccant of leguminous seed crops. Now Breese & Wheeler (45) have demonstrated its ability to kill selectively *Galium aparine* and other weeds of cereals that are resistant to the auxin herbicides.

Dalapon is by far the most important herbicide of this group and is employed in many parts of the world for the control of both seedling and established perennial grasses, particularly *Agropyron repens, Cynodon dactylon, Phragmites communis,* and *Imperata cylindrica.* It has little contact toxicity and is translocated in the plant in much the same way as 2,4-D [Wilkinson (345)]. The toxic symptoms are often indistinguishable from those produced by TCA. Sometimes the formative effects are insignificant and obviously indirect. It may be that they are the result of the known interaction between IAA and dalapon or TCA (271, 345). The main site of action of dalapon is in the leaves [Olsson & Salisbury (222); Wilkinson (345)]. Both chemicals cause marked changes in the chemical composition and metabolism of leaves [Corns (64); Dewey *et al.* (76); Miller & Corns (205); Rebstock *et al.* (245)].

Dalapon might be expected to act as an anti-metabolite to pyruvic acid and to inhibit enzyme systems using pyruvic acid as a substrate. Redemann & Meikle (247) have tested this hypothesis with pyruvate oxidase and carboxylase, but their results are not convincing and do not indicate that the blocking of pyruvic acid metabolism is the primary cause of the herbicidal action of dalapon. The growth of yeast can be inhibited by dalapon and Hilton *et al.* (141) have shown that this inhibition can be partially reversed by high concentrations of β-alanine. Similar experiments with germinating seeds were less decisive, but the utilization of β-alanine was affected, and the authors suggest that its metabolism may be involved in the mechanism of action of dalapon.

Slight changes in the substituents of the molecule influence both phytotoxic and selective activity [Poignant (235, 236); Poignant & Richard (237)]. The 2,2,3-trichloropropionic acid is similar in action to dalapon but less toxic [Barrons & McQueen (25)] while the sodium 2,3-dichloro*iso*-butyrate will kill annual monocotyledonous weeds, such as *Digitaria sanguinalis* and *Setaria* spp. in perennial lawn grasses. Some of the phytotoxic properties of both 2,4,5-T and dalapon are combined in erbon [(2,4,5-trichlorophenoxyethyl)-2,2-dichloropropionate] which is being tested as a nonselective soil sterilant [Swezey (297)].

Herbicides Containing the Amide, Thioamide, or Amidine Group

All the important new herbicides, with the exception of those already considered (auxin herbicides and substituted acetic acids), and endothal (3,6-endoxohexahydrophthalic acid), contain the amide moiety N — C = O

or one of the related groupings N — C = S or N — C = N. In Table II these herbicides have for convenience been classified in accordance with this aspect of their chemical structure. However, the physiological significance of these groupings and their relation one to another is not known, and the emphasis placed upon them for the sake of classification does not imply that they are necessarily associated with the modes of action of the compounds. Nevertheless, the fact that practically all the new herbicides do contain this similarity in chemical constitution is surely more than a coincidence.

Amides.—(*a*) 2-chloroacetamides. The relationship between chemical structure and toxicity to germinating grass species has been reported in detail by Hamm & Speziale for variations in the amide (120) and the acyl moieties (121). Both N-mono- and N,N-disubstituted compounds are phytotoxic to grass species and a three-carbon chain fulfills most of the requirements for maximum activity. N,N-disubstitution appears superior to N-monosubstitution with straight chain aliphatic groups but with branched chains the N-monosubstitution is the more active. Some heterocyclic derivatives are also highly active. With the acyl moiety a single α-halogen atom other than fluorine is necessary but replacement with other functional groups nullifies activity. Extension of the acyl moiety to include halogenated propionamides, as in the N,N-diallylamide of 2,2-dichloropropionic acid, and in butyramides, results in complete inactivity.

The two herbicides that have been marketed, 2-chloro-N,N-diallylacetamide (CDAA)(I)[4] and 2-chloro-N,N-diethylacetamide (CDEA)(II), are used for the control of grass weeds in maize, soyabean, groundnut, and several other crops. The diallyl compound has proved the most effective in field trials. It is applied as a pre-emergence treatment at rates of three to six pounds per acre and needs moist soil conditions for its full effect on germinating seeds [Crafts (69)].

Little is known concerning the mode of action of these compounds. Wangerin (325) claims that resistant plants absorb CDAA but metabolise it to glyoxylic and glycollic acids completely within a few days. Jaworski (150) studied the effect of CDAA on the respiration and growth of germinating seeds of a susceptible plant (ryegrass) and a moderately susceptible plant (wheat) and concluded that, in both, CDAA inhibited certain sulphydryl-containing enzymes and possibly oxidative phosphorylation. The R.Q. of wheat was increased but that of ryegrass was markedly decreased.

(*b*) N-(1-naphthyl)phthalamic acid (NPA) (III). This herbicide is used as the acid, the sodium salt and the imide; a summary of the relevant literature has been published (94). NPA is used primarily as a pre-emergence treatment for the control of germinating weeds in cucurbitaceous crops. The acid and sodium salt are recommended for cucumber, squash, pumpkin, cantaloupe, watermelon, and asparagus; the imide, which is less

[4] Roman numerals refer to Table II.

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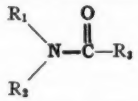
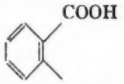
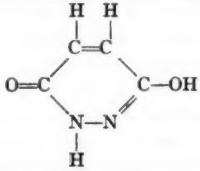
TABLE II

A CLASSIFICATION OF HERBICIDES CONTAINING THE AMIDE, THIOAMIDE, OR AMIDINE GROUP

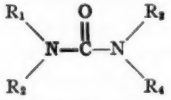
AMIDE GROUP

(A) Amides



	Common name	R_1	R_2	R_3	R_4
(I)	CDAA	$CH_2{:}CH{\cdot}CH_2{\cdot}$	$CH_2{:}CH{\cdot}CH_2{\cdot}$	$Cl{\cdot}CH_2{\cdot}$	
(II)	CDEA	$CH_3{\cdot}CH_2{\cdot}$	$CH_3{\cdot}CH_2{\cdot}$	$Cl{\cdot}CH_2{\cdot}$	
(III)	NPA		H·		
(IV)	Maleic hydrazide (MH)				

(B) Ureas



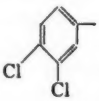
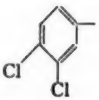
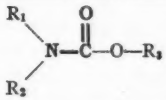
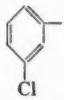
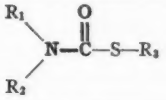
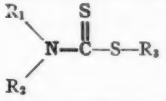
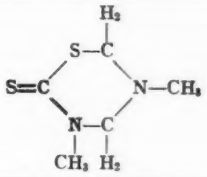
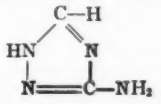
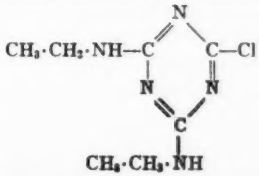
(V)	Dichloralurea (DCU)	$Cl_3C{\cdot}CH(OH){\cdot}$	H·	H·	$Cl_3C{\cdot}CH(OH){\cdot}$
(VI)	Fenuron		H·	$CH_3{\cdot}$	$CH_3{\cdot}$
(VII)	Monuron		H·	$CH_3{\cdot}$	$CH_3{\cdot}$
(VIII)	Diuron		H·	$CH_3{\cdot}$	$CH_3{\cdot}$
(IX)	Neburon		H·	$CH_3{\cdot}$	$CH_3{\cdot}(CH_2)_2{\cdot}CH_2{\cdot}$

TABLE II—*continued*

(C) Carbamates				
(X) Propham		H·	$CH_3 \cdot CH(\cdot CH_3)\cdot$	
(XI) CIPC		H·	$CH_3 \cdot CH(\cdot CH_3)\cdot$	
(D) Thiocarbamates				
(XII) EPTC	$CH_3 \cdot CH_2 \cdot CH_2\cdot$	$CH_3 \cdot CH_2 \cdot CH_2\cdot$	$CH_3 \cdot CH_2\cdot$	
THIOAMIDE GROUP				
(A) Dithiocarbamates				
(XIII) CDEC	$CH_3 \cdot CH_2\cdot$	$CH_3 \cdot CH_2\cdot$	$CH_2{:}CCl \cdot CH_3\cdot$	
(XIV) "Chem Vape"	$CH_3\cdot$	H·	Na	
(XV) "Vapam"	$CH_3\cdot$	H·	$Na \cdot 2H_2O$	
(B) Thiadiazines				
(XVI) "Mylone"				
AMIDINE GROUP				
(A) 1,2,4-Triazoles				
(XVII) ATA				
(B) *s*-Triazines				
(XVIII) Simazin				

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soluble and therefore more persistent in the soil, is recommended for potato and sweet potato [Crafts (69)]. Some weeds (*Amaranthus retroflexus, Xanthium* sp.) are more easily killed by foliar sprays (94) although the main effect of the herbicide is on the roots of seedlings. It causes many abnormalities in growth such as dwarfing, leaf malformations, and epinasty, but its most characteristic action is the inhibition of geotropism and phototropism [Mentzer & Nétien (203); Teas & Sheehan (300); Tsou *et al.* (310)].

It is doubtful if the inhibition of tropic responses is of major importance in the explanation of the toxic action, but this property has attracted the most attention. Mentzer *et al.* (202) thought that a close spatial relationship of the peptide bond and the carboxyl group was required for the inhibition of geotropism. The effects on geotropic and phototropic responses take place at concentrations below those that affect growth, and the correlation between straight growth and curvature is upset in roots and shoots of maize and pea, but not in oat coleoptiles (310). It has been suggested that the effect may be due to an interference with the lateral transport of IAA, but attempts to abolish the anti-geotropic activity of NPA by addition of IAA were unsuccessful (160). Many chemicals besides NPA interfere with tropic responses. Jones *et al.* (160) have reviewed the literature and reported results with a wide range of compounds (158, 159). Results for 2,4-D are given by Henderson & Peterson (138) and Audus & Brownbridge (17), for 2,4,6-trichlorophenoxyacetic acid by Brumfield (49) and for 2,3,6-TBA by Vander Beek (313).

(*c*) 1,2-dihydropyridazine-3-ol-6-one (MH, maleic hydrazide) (IV). This compound has been available commercially since 1948, and its use as a herbicide was reviewed by Crafts (65). Although physiologically an interesting compound and useful for the inhibition of sprouting, the prevention of bolting and the slowing down of the growth of many species, it has very limited application as a herbicide. In the United States it has been found successful for the control of perennial grasses, wild onion, and wild garlic, provided the land is ploughed or rotovated within four to eight days after treatment. The suggestion that it could be used for the sterilisation of the seeds of wild oats growing in barley and wheat has not proved practicable.

The literature relating to the physiological effects of MH is extensive and will not be fully covered here as it is only indirectly concerned with phytotoxicity. Pilet (234) reviewed the position in 1956, and a summary of the literature has been published (357). Theories have often related its mode of action to that of endogenous auxins. It has been classified as an anti-auxin, but as Audus & Thresh (19) point out, the claim that it enhances IAA oxidase activity [Andreae (9)] suggests that the antagonism is less direct than that required for a true anti-auxin. The increase in endogenous IAA obtained after treatment with MH by Audus & Thresh is contrary to

the findings of Kulescha (175) and Pilet (234) and is probably due to secondary effects of the chemical. The suggestion made by Muir & Hansch (215) that MH combines with free and physiologically essential thiol groups now seems unlikely in view of the fact that the molecule probably exists in the enol form and is therefore incapable of addition to a thiol group [Weller *et al.* (340)]. Furthermore, the latter authors have failed to find any evidence of an enzyme system capable of catalysing the reaction of the thiol radical with MH or converting MH into a compound that would add to a thiol group. The suggestion that MH inhibits growth by blocking dehydrogenase activity [Isenberg *et al.* (148)] is based on circumstantial evidence concerning the failure of tissue treated with MH to produce the typical tetrazolium reaction [Currier & van der Zweep (72)] but needs further investigation. The most interesting developments are perhaps those concerned with the interrelations of MH and gibberellic acid. Brian & Hemming (46) have suggested that the rapid growth of tall peas is due to a greater capacity to synthesise a postulated 'gibberellic acid-like hormone' and that the inhibition of growth caused by MH is due primarily to the blocking of this hormone.

Substituted ureas.—Dichloralurea (V) was the first of the substituted ureas to be recommended for weed control and, although of comparatively low toxicity, it has been found useful for the control of weeds, particularly grasses, in sugar beet, cotton, onion, and cucurbitaceous crops [Crafts (69)]. The phenylureas, fenuron (N,N-dimethyl-N′-phenylurea) (VI), monuron (VII), diuron (VIII), and neburon (N-butyl-N′-(3,4-dichlorophenyl)-N-methylurea) (IX) are much more phytotoxic and of greater commercial importance. These four herbicides are all relatively insoluble, persistent in the soil, and toxic to most species. They are, in consequence, used primarily as 'total' herbicides for the control of all types of vegetation growing in areas that are not intended for crops. A few species are partially resistant, but selectivity, where it is obtained, is usually dependent on the chemical not reaching the roots of the crop. The inherent phytotoxicity of monuron and diuron, as measured in nutrient culture, is about equal, and substantially higher than that of fenuron, while neburon is intermediate.

Movement in the soil is closely related to solubility which decreases from 2400 p.p.m. at 25°C. for fenuron, to 230 for monuron, 40 for diuron and only 4.8 for neburon. Of the four chemicals, monuron and diuron are the most extensively used, the former as a soil 'sterilant' in areas of average rainfall and the latter in regions of very heavy rainfall. As a selective herbicide, monuron shows promise in orchards and vineyards and is being used in asparagus, spinach, grass seed crops, citrus, and cotton. Because of its lower solubility, diuron may be preferable in cotton.

Studies on the mode of action of these herbicides have been primarily concerned with monuron. Early observations on the toxic symptoms caused by this chemical soon established: (*a*) that it had little effect on germinating

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seeds until the shoots had emerged above the ground; (*b*) that the first symptoms of toxicity were wilting and chlorosis [Christoph & Fisk (58)]; (*c*) that it entered the roots very rapidly but scarcely penetrated the leaves, and that movement was almost wholly in the xylem and controlled by transpiration [Fang *et al.* (91); Haun & Peterson (127); Minshall (206); Muzik *et al.* (216)]. It was also discovered that excised (216) and intact [Minshall (208)] roots could withstand concentrations that would be lethal to whole plants. Minshall (208) established the concentrations in nutrient culture that were toxic to root and shoot growth of different species and concluded that at concentrations of less than 32 p.p.m. the herbicides had little effect on the roots and that the site of action was in the leaves. When there was a rapid build-up in the leaf tissue to concentrations of more than 100 μg./gm. of fresh weight, then "water-soaked necrotic blotches" appeared within hours, but if the build-up was slower, extending over several days, then 50 μg./gm. of fresh weight could cause other toxic symptoms that also resulted in death. The influence of light on the development of toxic symptoms [Minshall (209)], as well as the inhibition of dry matter production in excised bean leaves [Minshall (207)] and sugars in legumes [Cooke (61)] had also been observed, but it was Wessels & van der Veen (341) who first reported the specific effects of these herbicides on photosynthesis and the Hill reaction. These observations have since been confirmed by Cooke (62) and Spikes (286). Both the rate-limiting photochemical and the dark phases of the Hill reaction seem to be involved, but the photochemical reaction is the more sensitive and is inhibited at concentrations of about $10^{-7}M$. It seems fairly certain, therefore, that the primary toxic effect of these substituted ureas is caused by their absorption and concentration on active sites in the chloroplasts and the consequent inhibition of photosynthesis. These effects can occur at concentrations of monuron of 5 to 15 μg./gm. of fresh leaf [Minshall (207)] and are probably the main cause of phytotoxicity under field conditions, although higher concentrations in leaves and roots will, no doubt, cause other phytotoxic symptoms.

Although more than a thousand substituted ureas have been screened for herbicidal activity [Abel (1)] little has been published. Thompson *et al.* (304) recorded the toxicity of a number of urea derivatives and Anderson *et al.* (8) have tested 12 nitrophenylureas. In general the asymmetrically substituted ureas are more toxic than the symmetrical compounds. For example, the symmetrical diphenylurea, one of the growth factors of coconut milk [Steward & Shantz (291)], is ineffective while phenylurea and N,N-diphenylurea are slightly active [Freed (99)]. Substitutions on the aromatic nucleus and the nature of the alkyl substituents have been investigated both for herbicidal activity [Anderson *et al.* (8); Freed (99)] and activity in the Hill reaction [Wessels & Van der Veen (341)]. Although the latter is indicative of the former, no close correlation exists.

Carbamates.—Since the last review little has been published on the mode of action of propham (X) (*iso*propyl-N-phenylcarbamate) or CIPC (XI). Shaw & Swanson (274) have reported on the phytotoxic properties of a series of more than 40 compounds. Mitchell *et al.* (210) have shown that although propham and CIPC are not readily transported from leaves, certain carbamates that contain the lactic acid structure, such as (1-carboxymethyl)-3-chlorophenylcarbamate, move through young barley leaves.

CIPC causes a decrease in root respiration [Swanson *et al.* (296)], an increase in sugars [Meade & Kuhn (199); Tomizawa & Koike (306)] and a marked effect on β-amylase [Moreland & Davis (212)].

Many thiocarbamates and dithiocarbamates are phytotoxic; some possess true auxin activity [Fawcett *et al.* (93); Van der Kerk *et al.* (314); Veldstra (319)], but none as yet combines both properties. One of the toxic carbamates tested by Shaw & Swanson (274) was *iso*propyl-N-phenylthionocarbamate, and herbicidal activity has been claimed for the salts of thiocarbamic acid [Szabó *et al.* (299)].

The carbamates that have been recently introduced as herbicides are:

(*a*) Ethyl-N,N-dipropylthiolcarbamate (EPTC) (XII). This is a pre-emergence herbicide first released for trial in 1956 that seems promising, at doses of about five pounds per acre, for the control of many annual grasses and some dicotyledonous weeds in lucerne, asparagus, and several vegetable crops [Antognini *et al.* (14)]. It does not inhibit germination but is toxic to seedlings causing a preliminary darkening in colour that is followed by a necrosis. This effect progresses downwards from the tips of the cotyledons. Leaves are trapped in their sheaths, and there are formative effects which are probably associated with a marked change in the waxy surfaces of the leaves.

(*b*) 2-chloroallyl-N,N-diethyldithiocarbamate (CDEC) (XIII). CDEC has proved of value for the control of annual grasses and a few other weeds in crops such as maize and soyabean. Its mode of action is not known, but the relationship between chemical structure and herbicidal effect has been studied in detail by Hamm *et al.* (119). It is interesting to note the similarity in chemical structure between several of the compounds tested by these workers and the dithiocarbamates shown to possess auxin activity by van der Kerk *et al.* (314).

(*c*) Sodium N-methyldithiocarbamate (XIV). This compound and the dihydrate (XV) [Hardman & Shafer (125)] are different from CDEC in their action as they decompose on contact with water to form toxic vapours. They are applied at rates of about 300 pounds per acre and under favourable conditions will kill deep-rooted perennial weeds as well as soil fungi, bacteria and insects.

(*d*) 3,5-dimethyltetrahydro-1,3,5,2H-thiadiazine-2-thione (XVI). This chemical is another soil fumigant that acts as a fungicide and nematicide

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as well as a herbicide. Its action is probably similar to that of the dithiocarbamates, as in warm moist soil it readily breaks down in the first instance to N-methylamino-S-methyldithiocarbamate (13,167).

3-amino-1,2,4-triazole (ATA) (XVII).—This comparatively new herbicide was originally introduced as a defoliant for cotton [Hall *et al.* (118)] but has subsequently been found to be toxic to many plant species and is now used to control several agricultural weeds, particularly *Agropyron repens* and *Cirsium arvense*, and also some aquatic plants.

The ease with which ATA is translocated throughout plants, following absorption by either root or shoot, has already been mentioned. This property permits it to be used for the control of deep-rooted perennials, and its rapid loss from soils allows it to be applied only a few weeks before susceptible crops are sown.

The first toxic symptom is usually the development of young leaves devoid of chlorophyll; this is followed by a localised breakdown of the chlorophyll in the older parts, the formation of anthocyanin pigments, desiccation, and death. The extent and duration of the chlorotic symptoms depend on the dose applied; if sublethal amounts are used the chlorotic tissue may eventually regain its colour. When woody plants are sprayed in the dormant season, the chlorotic symptoms may not develop until growth starts again in the spring. Ennis (83) has also shown that if maize is treated when the cob is maturing then the seed that is produced may give rise to chlorotic seedlings.

Several other compounds cause similar chlorotic symptoms, but few are especially toxic in other ways [see review by Currier (71)]. The mode of action of these substances is not known, but it has recently been shown that the chlorotic symptoms produced by the amino acid analogue DL-O-methylthreonine can be prevented by the simultaneous application of two to five times as much DL-*iso*leucine [Gray & Hendlin (111)]. The superficial similarity in structure between ATA and pyrrole has led to the suggestion that ATA interferes with chlorophyll synthesis by blocking porphyrin formation, but there is no experimental evidence for this. ATA might chelate Mg in the plant although it does not do so readily *in vitro*. Menoret & Tracez (201) have, in fact, prevented the development of chlorotic symptoms in treated tomato plants by adding $MgSO_4$ to the soil in which the plants were growing, and Hall & Johnson (117) have hastened the resynthesis of chlorophyll in chlorotic tissue by the addition of Mg.

Microscopic studies have established that one of the main effects of ATA is to inhibit the formation of plastids [Rogers (258); Pyfrom *et al.* (238)]. How closely these visible symptoms are associated with the intrinsic toxic action of ATA is not known. It has, however, been established that this chemical interferes with mitosis [Grigsby *et al.* (114)], that it affects tissue metabolism immediately after entry and causes an initial stimulation of respiration [Miller & Hall (204); Herbert & Linck (139)] and, perhaps

most important, that it has a specific effect on catalase in both animals and plants [Heim *et al.* (133)]. This depression of catalase activity apparently occurs whenever ATA is present in the tissue, but as the chemical does not persist for long, the catalase activity returns to normal after a few days even though chlorophyll production remains inhibited and the chlorotic symptoms still persist (238).

s-*Triazines.*—If the three reactive chlorines of cyanuric trichloride are replaced by one, two, or three low molecular weight alkylamino, alkoxy, or alkylthioxy groups, an extensive series of phytotoxic chemicals can be produced differing in their herbicidal characteristics. Many of these chemicals have been tested for herbicidal activity [Gast *et al.* (108); Gysin & Knüsli (115, 116); Koopman *et al.* (171); Koopman (170)] and already one extremely effective herbicide has emerged. Simazin (XVIII) (2-chloro-4,6-*bis*(ethylamino)-*s*-triazine) is active only when applied to the soil, where it is very persistent and highly toxic to most species. It is taken up by the roots, moved in the transpiration stream, and concentrated in the leaves where it affects photosynthesis and kills the plants in much the same way as the substituted ureas. Its chief use is as a soil 'sterilant,' but it is relatively nontoxic to a few crop plants, particularly maize (115, 116).

Chloro-*bis*amino-*s*-triazines with one or two secondary amino substituents in the triazine ring, such as trietazin (2-chloro-4-ethylamino-6-diethylamino-*s*-triazine) and chlorazin (2-chloro-4,6-*bis*(diethylamino)-*s*-triazine), are more selective and can be used on pea, tobacco, potato, and some vegetable crops (115, 116). The dichloro-*s*-triazines are relatively unstable especially if they contain aliphatic substituents (116), but some may prove to be effective herbicides (170). Among the *s*-triazines in which all the chlorines have been substituted, the most interesting are the 2-alkyl- and the 2-alkoxy-4,6-*bis*(alkylamino)-*s*-triazines and the *tris*(alkylamino)-*s*-triazines. The two former are more soluble than their corresponding chlorinated analogues and are toxic to maize (116).

Simazin is readily absorbed by maize but no measurable quantity can be detected in plants that have been treated with doses equivalent to 10 kg. per hectare. It has been suggested (116) that the resistance of maize is due to a detoxication mechanism which hydrolyses the simazin to 2-hydroxy-4,6-*bis*(ethylamino)-*s*-triazine as soon as it enters the plant. Extracts of maize have been shown to decompose simazin and to lose this property if heated to 80°C. for two hours, but similar extracts prepared from wheat had no effect [Roth (264)].

Conclusion

This review is concerned primarily with herbicides developed during the last twelve years. The older herbicides such as chlorates, arsenites, borates, and dinitroalkylphenols are not considered, nor is the wide range of phytotoxic chemicals that still await assessment as herbicides. The large number of references that it has been found necessary to quote gives an

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indication of the magnitude of the research effort being put into studies on the mechanisms of herbicidal action; yet we still do not understand the exact manner in which any herbicide exerts its toxic effects. This is perhaps not surprising when one considers the limitations of our knowledge of the physiological and biochemical processes in the normal living plant. Without a fuller knowledge of the growth processes of weeds and crops no proper understanding of the toxic action and selectivity of herbicides is possible.

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MORPHOGENESIS IN LOWER PLANTS[1,2]

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The lower plants attract the developmental physiologist with a bizarre variety of systems in which individual, normal, developing cells can be easily and directly observed and experimented upon. For the present purposes, this group includes the slime molds, true fungi, algae, and bryophytes. Only a fraction of the current literature can even be mentioned in the space available here.

Nucleocytoplasmic relations.—Enucleate parts of *Acetabularia,* etc., survive for several months, normally regenerate the complex apical whorls and cap, occasionally regenerate a basal rhizoid [Hämmerling (1)], and carry on a large net synthesis of both proteins and nucleic acids for several weeks [Brachet *et al.* (2)]. However, grafts in which the nucleus comes from one species and the bulk of the cytoplasm from another, while first developing caps of an intermediate character, ultimately are governed by the nucleus. Evidently the cytoplasmic carriers of nuclear information are relatively long lived in the Acetabulareae; no wonder, considering that an adult cell of the order of 10 mm.[3] probably remains diploid (1). There is evidence of cytoplasmic control of nuclear functions; in polynuclear forms made by grafting, the rate of protein synthesis is unaffected but the volume of the individual nuclei decreases [Werz (3)]. Moreover, when young nuclei are grafted into old cytoplasm they are induced to initiate prematurely the mitoses preliminary to gametogenesis. In weak light, this condition which stimulates mitosis develops without whorl or cap formation [Beth (4)].

The carriers of cap character information produced by the basal nucleus are somehow accumulated in the apical region where caps form (1). A preliminary ultraviolet inactivation study weakly suggests that these carriers are nucleic acids [Six (5)].

Waris (6, 7) and Kallio (8, 9, 10) have reported their studies of desmids of the genus *Micrasterias.* The wild type semicell consists of a centrally located end lobe flanked by two "wings," each of which consists of two side lobes. This structure is cut by two planes of symmetry and is called biradiate. Enucleate forms were obtained by centrifugation at metaphase for the subsequent cytoplasmic division yields an enucleate and a binucleate semicell. The former does not normally survive more than one day but in this period it grows a new semicell whose form, while simplified, has the

[1] The survey of literature pertaining to this review was concluded in August, 1957.

[2] This work was materially assisted by the Office of Naval Research under contract Nonr 1677(02)NR 164-406.

symmetry of its parent. Thus, an enucleate biradiate wild type semicell with five lobes produces a new semicell with five or three lobes and likewise of biradiate symmetry. An enucleate "defect mutation" semicell—one lacking one wing and, hence, consisting of three lobes cut by one plane of symmetry—produces a new semicell with one or two lobes and likewise of "uniradiate" symmetry [Waris (6)].

Of particular interest is the evidence developed by these workers which suggests that the cytoplasmic determinants of these lobe patterns are immobile primordia which perpetuate some information which is independent of the nucleus. The most suggestive observation is this: the uniradiate defect mutations were observed to arise from the wild type only three times in thirteen years of study [Kallio (9 p. 121)]. Nevertheless, uniradiate semicells sometimes give rise first to a single biradiate semicell which breeds true and subsequently to a series of uniradiate semicells which breed true [Kallio (8, p. 86)].

Moreover, there is graphic evidence that new lobe primordia arise most easily through the splitting of an old primordium rather than *de novo*. Thus, Kallio (8 p. 86) reports that the commonest mode of spontaneous back mutation of a uniradiate form yields, after one or more generations, cells in which an original uniradiate mother semicell is joined to a biradiate semicell bearing two wings which are in a plane perpendicular to that of the mother semicell.

Discovery of a remarkable new species of desmid, *Scottia mira* [Grönblad & Kallio (11)] may extend the possibilities of desmid study to the problem of polarity; for this species is unique among desmids in that each cell consists of two semicells of distinctly different shapes. However, all the observations appear to have been made upon dead specimens, so there is some doubt as to the stability of *Scottia* and its mode of multiplication.

Diploid forms of *Micrasterias,* produced by metaphase centrifugation and other means show the tendency, long established in other forms, to maintain an approximately fixed nucleocytoplasmic volume ratio. In some cases the diploid cells expand chiefly by enlarging their wings; but in others, the wings multiply so as to produce forms with three or four wings of normal size [Kallio (8, 9, 10)].

Bauer (12) has studied the development of a diploid protonema derived by regeneration from a young sporophyte of the moss, *Georgia pellucida.* It passes through the two filamentous phases characteristic of the normal haploid protonema (see p. 364), and except for having the larger cells typical of chromosome doubling it is morphologically indistinguishable from the haploid filaments. However, its subsequent behavior is remarkable. Under proper conditions—these include staling of the medium and the use of 3 per cent agar as opposed to 1 per cent agar—the protonema regularly develops numerous buds which grow directly into sporophytes; the leafy gametophore stage is completely bypassed. While clear-cut control experiments with haploid protonemas are not reported, such vegetative sporophyte development was apparently never observed to occur in haploid

filaments by either Bauer or a number of earlier workers. The vegetative sporophyte only occasionally developed to the point of forming spores, but those spores that did develop were capable of germinating into filaments indistinguishable from normal haploid ones.

These results appear to offer a choice of two interesting conclusions: either a minimum of two chromosome sets is required for sporophyte development in *Georgia,* or protonemas derived by regeneration from the sporophyte retain some subtle cytoplasmic factor which is both essential for sporophyte development and lost during sporogenesis.

Germination.—Forsyth (13) and, more clearly, Allen (14) demonstrated that *Puccinia graminis* uredospores emit a somewhat volatile self-inhibitor of germination. Emission occurs during approximately 5 hr. after floating the spores in water and then ceases (14). The inhibitor is not CO_2. (Forsyth's claim to have identified the inhibitor as trimethylethylene is unconvincing. It is based primarily upon a very crude similarity in the ultraviolet absorption spectra of an alleged inhibitor solution and a trimethylethylene solution.) A self-stimulator of germination emitted by uredospores was recently discovered by Allen (15) and by French *et al.* (16); it inhibits germ tube elongation. This stimulator likewise proves to be volatile and probably has at least two components, one of which has been definitely identified as pelargonaldehyde. However, the capacity of pelargonaldehyde to stimulate germination is also found to a comparable degree in a variety of aldehydes and alcohols of approximately the same molecular size. It is of great interest that pelargonaldehyde likewise induces differentiation of the germ tubes into structures resembling appressoria, infection hyphae, infection pegs, and substomatal vesicles [French *et al.* (16, 17, 18)].

Origin of polarity.—At relatively low concentrations of amoebae of *Dictyostelium discoideum,* aggregation begins with the appearance of small regions in which the amoebae are relatively concentrated, a process of centripetal movement then spreading out from these centers [K. B. Raper (19)]. It would be very difficult to avoid the conclusion that the cells in these primordial centers, called initiators by Sussman (20), are subtly different from the others. Sussman (20) has reviewed a series of studies on the origin of these initiators. He has concluded that at the end of the growth period the fraction of initiator cells is fixed. (The effective fraction is supposed to depend upon the sensitivity of responder cells under the given conditions.) Shaffer (21), on the other hand, concludes that a large fraction of the myxamoebae may act as initiators; the centers of aggregation form around those few which happen to develop this capacity first.

This reviewer finds Shaffer's view preferable: Sussman supports his view with the results of a fluctuation test (22). Small numbers of myxamoebae were confined within areas of about 4 mm.^{2} and at cell concentrations otherwise compatible with aggregation. After an interval found to be more than adequate for aggregation in large groups (130 to 500 mm.^{2}), a large fraction of the replicates of this experiment (with small groups) failed to aggregate. However, the conclusion that the nonaggregating

groups lacked initiators is invalidated by a technical mistake; the amoebae were confined by some mysterious property of the agar substratum which underlies a recently adsorbed droplet of suspended amoebae. This property was surely no barrier to the diffusion of acrasin, etc; hence, the concentration of acrasin, etc., in these small groups must have been greatly reduced as compared to cultures on areas of 130 to 500 mm.^{2}. Now in these latter, a reduction of the cell concentration to about one-half to one-seventh of those used in the small groups completely prevented aggregation, suggesting that a reduction in the concentration of acrasin, etc., to the degree that must occur in small groups could well block aggregation. Sussman presents a second argument: in large groups, the average number of aggregations per cell were plotted against cell concentration. The curve shows a rather sharp maximum of one aggregate per 2100 cells at the "optimal" density of 200 cells/mm.^{2}. Now, where the small groups had included a total of about 1000 cells per group, about two-thirds of the aggregates lacked centers. If this lack really arose from the chance absence of initiators, then the Poisson equation would yield the fraction of initiators. The result was one initiator per 2300 cells. It was assumed that at the "optimum" density in large groups the number of aggregates per cell equalled the number of potential initiators per cell. If this were true, then the agreement between the two numbers would strongly buttress Sussman's conclusion. However, the implications of the above assumption are extremely difficult to swallow: (*a*) The average aggregation size rises rapidly on both sides of the optimal density. Hence, Sussman's assumption implies that the two mechanisms which rapidly increase the aggregation size at sub- and super-optimal densities must both become virtually inoperative at the optimal point. (*b*) If wild type cells are mixed with aggregateless mutants, one can obtain up to one aggregate per 24 cells, depending upon the mutant (23). Yet, an ingenious study of wild type and aggregateless mutants separated by thin agar membranes confirms what are in any case the simplest assumptions (24): (*i*) The initiator cells in the mixture come exclusively from the wild type component; (*ii*) Aggregateless cells are, if anything, less responsive to acrasin than wild type cells. In the face of these latter facts, the assumption of equal aggregate number and initiator number requires that either (*i*) or (*ii*), or both does not hold when the two cell types can come into direct contact. In conclusion, it is suggested that critical evidence may be obtained by repeating the fluctuation test with cells confined by a diffusion barrier.

The early development from carpospores or tetraspores of diverse red algae involves the formation of a cushion of cells lacking any apparent polarity [Fritsch (25 p. 607)]. Jones (26) reports that in *Gracilaria verrucosa,* a cushion developer, polarity, in the form of apical initials, only rarely develops except in "rafts" derived from the coalescence of cushions from two or more spores. Is this yet another surface/volume effect?

Various investigators have induced a more or less prolonged apolar phase into the development of bryophytes (27 to 30). Thus, Allsopp (30)

sowed spores of two species of liverwort on a medium containing 2 per cent glucose. Some of the cultures developed amorphous tissues consisting of largely apolar, undifferentiated, and rapidly growing cells which continued in this state for at least six months. Upon transfer to glucose-free media, normal tissues rapidly developed.

In the normal development of various mosses (31), of *Dictyopteris* (32) and various Fucaceae (33) among the brown algae, and of diverse phycomycete conidiospores (34, 35), *Botrytis* conidiospores (36), *Puccinia* uredospores (37) and basidiospores (36) among the fungi, germination immediately yields a more or less polar structure whose axis can be determined by unilateral light and other gradients applied to the developing spore or zygote. There are many scattered reports of low percentages of bipolar or even multipolar germination in such forms. However, it is not clear whether even approximately normal eggs or spores could so respond. In some cases, the multipolar forms may have arisen from multicellular zygotes or spores arising from the fusion of normal eggs or spores. Thus Whitaker (39) finds that *Fucus vesiculosus* eggs, if raised at excessively high temperatures, often fail to separate from some of their sister eggs in the öogonium; the resultant fusion forms frequently develop in a multipolar manner, though the number of rhizoidal outgrowths initially formed never exceeded the number of constituent cells.

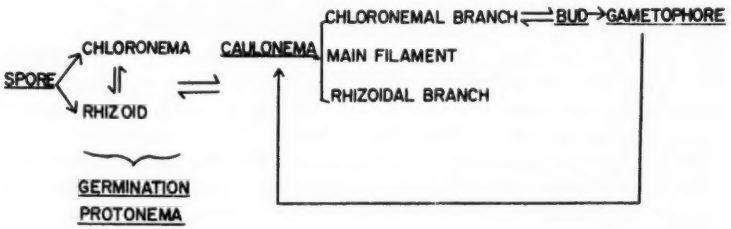
On the basis of these facts, it was possible to entertain the thought that the light or other directive influence in these various cases does not direct a purely epigenetic development of polarity but causes the rotation —to present an extreme alternative—of some single preformed asymmetric and determinative structure such as the nucleus. Indeed this last has been reported to occur within certain green algae zoopores after attachment to a substratum [Kostrum (40)].

Recently, however, it has been found that *Fucus furcatus* zygotes respond to illumination with plane polarized white light by growing out at approximately right angles to the direction of illumination, and with a strong tendency to be in the plane of vibration of the electric vector [Jaffe (41, 42)]. It is clear that every cell is here being stimulated in a symmetric manner, that is, to grow out in both of two opposite directions. In response, up to half the zygotes produce bipolar forms, the rest developing in one direction or its opposite but not both. In this case at least, it seems clear that the polarity arises in some more epigenetic manner than through the directed rotation of some preformed asymmetric structure.

The above discussion concerns the origin of apicobasal polarity. Study of the origin of dorsiventral polarity, using liverwort gemmae, began long ago; to my knowledge, Mirbel's 1835 paper on this subject (43) is the earliest study of the origin of polarity in any form. Since the polarity of the developing gemma is readily determined by various external influences, these gemmae have been recently considered as being comparable in their organization to *Fucus* eggs, moss spores, etc. However, the careful work of Fitting (44), his student Halbsguth (45, 46) and "grand-student"

Kohlenbach (47) clearly show this interpretation to be misleading. The external directive agents are not acting to establish dorsiventral polarity *de novo*. Rather they act to favor one or the other of two competing meristems which have a preformed tendency to lay down lobes with opposite dorsiventral polarities: A gemma is a disc-shaped structure with two oppositely placed notches in its edge. These notches are the site of the two apical meristems whose activity primarily constitutes germination. Since each notch behaves independently, the further discussion will refer to a single notch and its associated tissue. Both flat outer layers of the gemma exhibit a preformed, visible and fixed ventral differentiation. Seen in microscopic section, each notch usually contains what can be more or less clearly seen as two apical meristems, each nearer one of the flat faces (46). Under illumination directed at one flat face, it appears that the meristem nearer to the light is relatively inhibited; the farther one becomes dominant and lays down a thallus lobe with a ventrally differentiated face extending the layer already present in the gemma and therefore lying away from the light, and a dorsally differentiated layer lying toward the light. But, under illumination directed equally at both flat faces, so-called isolateral forms sometimes develop. That is, both meristems develop to an equal degree, so a form with two ventrally differentiated layers lying toward the light develops. Dorsally differentiated tissue develops when the two meristems begin to diverge so as to lay down lobes diverging from that plane of symmetry which is parallel to the flat faces. This dorsal tissue develops to some extent internally, proximal to the crotch, and subsequently at the inside faces exposed by the splitting growth (44).

Origin of buds in mosses.—On the basis of the older work, together with more recent papers, particularly those of Wallner (48), Heitz (31, 49), Meyer (50), Sironval (51, 52), Fitting (53), Bopp (54 to 57), and Allsopp & Mitra (58), the following tentative scheme can be drawn:



An important feature of this scheme is that the protonemal stage which intervenes between spore and bud consists of two distinct substages: first what I will refer to as a germination protonema, and secondly the caulonema (48, 51, 56). The buds in most species form only upon the proximal cells of the chloronemal branches of the caulonema (58).

Although quantitative studies are lacking, the literature allows the following qualitative characterizations of the germination protonema: (*a*) The chloronema and rhizoid are extremes of a continuum of forms (51, 53). (*b*) A chloronema-generating filament apex can be converted to one generating a rhizoid and visa versa by changes in pH and salt concentration (53); clear evidence that mature cells can be so transformed is lacking. (*c*) Intergrades are somewhat less stable than more extreme forms of the chloronema-rhizoid continuum (50, 51, 53). Thus, (i) a spore commonly sprouts both a distinctly chloronemal and a distinctly rhizoidal filament (49, 50); (ii) Apex conversion [see (*b*)] may not occur until a few days after the causal environmental change and then occurs quite suddenly (53 pp. 654-55). (*d*) Point (c–i) also indicates that under many common conditions, either extreme form alone is in turn less stable than both together. This conclusion is buttressed by Fitting's claim that conversion from chloronemal to rhizoidal generation occurs relatively readily in germlings lacking any rhizoidal filaments (53 pp. 654–55).

The caulonema is generated by a transformed chloronemal apex. This transformation to a caulonema is favored by sunlight as opposed to light from tungsten filament bulbs, by the presence of growing *Penicillium* sp. hyphae, and by material from a degenerate chloronema [Sironval (51)]. The reverse transformation, from caulonema to chloronema, can be induced by isolating caulonemal sections from the culture in which they had developed. This holds for cells newly generated by the apical cell and probably also for cells formed before the isolation [Bopp (56)].

Bud formation is greatly stimulated by various 6-(substituted) aminopurines in concentrations of as little as 10^{-8} *M* [Gorton *et al.* (59)]. Which transformation is so hastened in the chloronema to bud sequence is uncertain. Bud formation is greatly inhibited and buds are induced to revert to the filamentous stage by 10^{-4} *M* indoleacetic acid [Bopp (57)].

Origin of reproductive structures, heterogenous chemical control.— Raper (60) has carefully reviewed the older literature. Hustede (61) has found some striking effects of β-indole derivatives on the development of reproductive structures in two green algae. As little as 10^{-8} *M* indoleacetic acid stimulates zoospore formation in *Stigeoclonium*. As little as 10^{-9} *M* indoleacetic acid or tryptophan likewise stimulates zoosporangial formation but inhibits gametangial formation in *Vaucheria*, while as little as 10^{-7} *M* indole, indolealdehyde, or indolecarboxylic acid has the opposite effect, favoring gametangia and opposing zoosporangia. Moreover, these latter, short-side-chain compounds tend to favor öogonia as opposed to antheridia.

Cantino has published on the development of *Blastocladiella*, a water mold metabolically notable for producing no net CO_2 at all (62 p. 333). He has found that if the bicarbonate concentration of the medium lies in a concentration range (.004 *M* to .01 *M*) slightly below that which completely

inhibits growth (.05 *M*), the zoospores are induced to develop into thick-walled resistant sporangia (R.S.) instead of into thin-walled sporangia (63). He believes that the bicarbonate acts by speeding certain carboxylation reactions. Most probably, however, the bicarbonate acts by lowering the extracellular or intracellular pH, or both. (*a*) In the experiment cited above, the medium was so poorly buffered that the 2½ fold range of R.S.-inducing CHO_3^- concentrations is associated with a twofold range of extracellular hydrogen ion concentrations. (*b*) Addition of bicarbonate to medium B, which is buffered with .013 *M* phosphate failed to induce R.S. formation (62 p. 333). (*c*) "Gaseous CO_2 at concentrations permitting growth never induced more than 50 per cent (average *ca.* 5 to 25 per cent) of a population . . . to form resistant sporangia" (62 p. 350). (*d*) "Extreme reduction of the atmospheric CO_2 concentration often induced the formation of large quantities (50 to 85 per cent) of R.S. plants" (62 p. 333).

Origin of reproductive structures, autogenous chemical control.—Gussewa (64) has demonstrated that zoospore production is set off when the respiration of the green alga, *Oedogonium* has raised the extracellular concentration of CO_2 to a few per cent in the gas phase. He showed that this is not an extracellular pH effect but pointed out that the CO_2 may well operate through an intracellular pH drop.

Studies of the hormonal initiation (and control) of sexual reproduction in the fungi have been carefully covered in recent reviews by J. R. Raper (60, 65). Since the time of these reviews, the important paper of Plempel (66) on *Mucor* has appeared. He has clearly demonstrated that sexual interaction in *M. mucedo* involves at least four stable substances: (*a*) the plus progamone; the filtrate from the isolated plus mycelium contains this agent as shown by stimulation of the minus mycelium to liberate a second substance; (*b*) the minus gamone; thi sagent induces the otherwise untreated plus mycelium to develop zygophores; (*c*) the complementary minus programone; (*d*) the complementary plus gamone.

Light requirements for development.—In general, low intensity developmental light requirements ($< 10^5$ ergs/mm.^{2}) are satisfied only by the blue part of the visible spectrum. Thus, blue light is required for fruit body maturation in *Coprinus lagopus* [Borriss (67); Bünning *et al.* (68)] at a dose of $\geq 10^2$ ergs/mm.^{2} [Stiefel (69)], and to speed sporophore initiation in *C. lagopus* at $\geq 10^{-1}$ ergs/mm.^{2} [Madelin (70)]; for sex organ initiation and development in *Pyronema confluens* (71), at $\geq 10^4$ ergs/mm.^{2} [Kerl (72)]; for the initiation of long conidiophores in *Aspergillus gigantens* at $\geq 10^4$ ergs/mm.^{2} [Gardner (73)]; for apothecium formation in *Ascophanus carneus* [Stoll (74)]; for trophocyst formation in *Pilobolus Kleinii* [Page (75)]; for öogonial formation in *Laminaria* [Harries (76)]; for rhizoid formation in *Spirogyra fluviatilis* [Weihe (77)]. Moreover, there is evidence that short wavelengths are more effective than long ones in induc-

ing the localization of conidiophores and conidia in those hyphal tips of *Fusarium discolor* which are young during light exposure; $\geq 10^2$ ergs/mm.^{2} are needed [Bisby (78)]. The alleged effect of red light in favoring sporangiophore formation in *Choanephora cucurbitarum* [Christenberry (79)] was probably due to the temperature of the illuminated plant which was 10°C. higher than the dark control; high temperature is found to favor sporangiophore formation [Barnett & Lilly (80)].

The only clear exception to the blue light rule is the initiation of fruit body formation in the acellular slime mold, *Didymium eunigripes* [Straub (81); Lieth (82)]. According to Lieth (82), the light requirement for fruiting can be met not only by $\geq 10^4$ ergs/mm.^{2} blue light but also by $\geq 10^5$ ergs/mm.^{2} red light; moreover, $\geq 10^4$ ergs/mm.^{2} of green light markedly inhibits the initiation of fruiting by a simultaneous dose of red light. Radiation lying between 350 and 390 mμ. is likewise reported to stimulate fruting (81).

It would not be surprising if these low intensity blue morphogenetic effects ultimately prove to be mediated by the same mysterious photoreceptor responsible for phototropism.

Borriss (67) made the remarkable discovery that mechanical stimulation will substitute for blue light in allowing fruit body maturation in *Coprinus*. Moreover, the same dark reaction seems to limit the effectiveness of both forms of stimulation [Stiefel (69)].

In various cases it has been observed that these blue light effects are restricted to illuminated regions of the plant and are not translocated (70, 71, 78, 83).

Origin of reproductive structures, other physical factors.—Hawker's (84) review can be consulted here. More recently, Sproston & Pease (85) have made the first observation of a thermoperiodic response in a cryptogam (formation of apothecial initials in *Sclerotium*). In a careful study, Plunkett (86) has established that the initiation of sporophore rudiments as well as the initiation of caps in *Polyporus* is markedly speeded by high transpiration rates brought about by dry air flow. Now there is evidence in the higher fungi (as well as the phycomycetes) that mycelial protoplasm is induced to flow to a region of low external water activity [Buller (87 pp. 85, 96, 123)]. Hence, Plunkett's paper suggests an experimental attack upon the often observed relation between protoplasmic accumulation and the initiation of primordia.

Neoplasms.—Thomas *et al.* (88) have shown that rose-comb disease of mushrooms bears notable resemblances to animal cancer. The disease which is induced with particular effectiveness by diesel oil fumes is characterized by large, typically cerebriform outgrowths from the cap. These tumors may greatly outgrow the normal sporophore tissues; they often lack any gill tissue differentiation; and they are made up of cells which have an ab-

normally large number of nuclei and abnormally high basophilia, and exhibit "endomitosis, multipolar spindles, chromosome aggregation, polyploidy, aneuploidy, and micronuclei."

Persistence of differentiation.—Recent work continues to provide evidence of the relative stability of polarity even in simple systems: (*a*) An individual myxamoeba of *Dictyostelium* when confronted with a suddenly reversed gradient of acrasin usually "makes a U turn without ever losing its elongate shape"; occasionally it balls up, at the same time sending out a pseudopod toward the acrasin [Bonner (89)]. (*b*) Myxamoebae stuck together in fabricated loops as well as in the previously observed naturally occurring ones [Shaffer (90)] stream in a constant direction, that is either clockwise or counterclockwise. Bonner (89) argues—I believe correctly—that such behavior cannot be explained by an extracellular chemical gradient. For if, at any one time, cells in one part of the loop are exposed to a clockwise gradient, then others at this same time must necessarily be exposed to a counterclockwise one. Moreover, there appears to be inadequate reason to postulate waves constructed of successive zones of (i) acrasin gradient stimulation, (ii) response via chemotaxis and acrasin secretion, (iii) refractory state, . . . etc., passing around the rings —a possibility implied in Shaffer's discussions. (*c*) Hyphae of *Saprolegnia* when exposed to a suddenly reversed gradient of a mixture of amino acids toward which they respond chemotropically, subsequently grow in a hairpin turn, although they also react in part by developing new laterals far in back of the tip (35). (*d*) In the absence of strong external counter gradients, isolated cells of *Cladophora* [see(91)], *Enteromorpha* (92), *Chara* and *Nitella* (93), and *Griffithsia* [see (91)] show at least a strong tendency to regenerate new shoots and rhizoids with unchanged polarity.

On the other hand, in the case of various cellular, filamentous forms with apical meristems, environmental changes can convert the shoot-generating apical cell to a rhizoid-generating one or visa versa. The older work of this sort on *Cladophora, Sphacelaria,* and various Florideae is reviewed by Bloch (91). An impressive example of this phenomenon is furnished by Fitting's study of certain moss protonemas (53). A chloronema transferred to media relatively lacking in salts or relatively alkaline began generating a rhizoidal filament at its tip within a few days, while a rhizoidal filament transferred to relatively acid media soon began generating a chloronemal filament at its tip.

Similarly, in various large coenocytes, *Bryopsis, Dasycladus* and *Caulerpa* as well as similar large multinucleate isolated internodal cells of *Griffithsia* (91), *Chara* and *Nitella* (93), the application of reversed gradients or other agents can reverse the polarity.

A number of workers have succeeded in forcing more or less differentiated gametangial cells to sprout vegetative hyphae. Kerl (*72*) induced such effects by surgically isolating gametangia of *Pyronema* from the rest

of the mycelium. All cells of all stages in gametangial development frequently sprout vegetatively upon isolation except for the mature ascogonial cell; i.e., the as yet undelimited primordia, the recently delimited gametangia, the mature antheridia, trichogynes, and ascogonial stalk cells all do so. The mature ascogonial cells, however, which may be likened to eggs, respond to isolation with numerous small outgrowths which suggest abortive ascogenous hyphae rather than vegetative ones—behavior resembling parthenogenesis.

Bistis has studied *coitus interruptus* in *Ascobolus* (65, 94). If the oidium, a sort of male cell which induces ascogonial development, is removed before the ascogonium has matured, the latter sometimes sprouts vegetatively from its tip. However, the differentiated cells of the mature reproductive structure (except for the stalk cells which "are indistinguishable morphologically from vegetative hyphal cells") do not so respond. Denffer & Hustede (95) induced young antheridial primordia of *Vaucheria* to continue their elongation in the form of vegetative filaments rather than maturing sexually, by treatment with as little as 10^{-9} *M* indoleacetic acid. Mature gametangia, however, do not respond to the IAA (see p. 365).

Raper caused young, undelimited öogonial primordia of a variety of homothallic species of the Saprolegniaceae either to drain their contents back into the parent hypha or sprout antheridial hyphae by treating them with crude concentrates of hormone A from *Achlya*. Once delimited, however, the öogonia no longer respond (96). Bopp (56) has succeeded in causing *Funaria* buds to revert to a filamentous form via 10^{-4} *M* indoleacetic acid, and *Funaria* caulonemal filaments to revert to the chloronemal form via isolation. Sandan (97) reports a remarkable transformation of a mature rhizoidal cell of *Nitella* into an internodal one as a result of isolation from the rest of the plant. This metamorphosis began about two months after isolation and yielded an internodel cell of about one-eighth normal diameter with a linear instead of a spiral white line and streaming pattern. It should be noted that, with the exception of the reports of Sandan and of Bopp, the various transformations reviewed here do not represent so much the metamorphosis of an entire mature cell from one differentiated state to another as a change in the cell type generated by an obvious meristem or at most the sprouting of a changed cell type from a restricted region of a partially differentiated cell.

Scherr & Weaver have reviewed the extensive literature on the dimorphism phenomenon in yeasts (98). A reading of this review as well as the discussion in *Henrici's* handbook (99), indicates that the transformation from the yeast phase toward the mycelial phase involves a failure to carry "cell division" to the point of cell separation, a lengthening of individual cells, and a tendency to restrict budding to the anterior ends of cells attached in chains [see (99), Fig. 116; see also (98), Plate II, Fig. 4, Plate V, Fig. 4, Plate VI, Fig. 1].

Evidently, the last two features may be considered as a change in cell polarity, but it is not clear from the published data whether these changes involve only cells formed in a new environment, or whether cells already formed are likewise changed. Moreover, the polarity changes are not quite as extreme as superficial examination suggests for Barton (100) carefully observed *Saccharomyces cerevisiae* and found that successive buds showed a strong tendency to be antipodal.

Intracellular loci and directions of wall extension.—According to Mitchison (101), about 90 per cent of cells of the fission yeast, *Schizosaccharomyces Pombé* elongate by growth restricted to one end. Moreover, studies of various isolated fungal hyphae have clearly indicated a similar restriction of growth in *Peziza* (102), in the stage I sporangiophore of *Phycomyces* (103), and in *Rhizopus* (34). Now, the shape changes in the budding of yeasts are incompatible with such tip growth (104). It may, therefore, be guessed that both the cell lengthening and the apical localization of budding which characterize the passage of dimorphic yeasts toward the pseudomycelial or mycelial phase are manifestations of an increasing restriction of growth to the apical pole of each cell.

Green (105, 106) has carefully studied the growth of the lateral wall of the enlarging internodal cell of *Nitella*. At least as averaged over short intervals of time, wall extension goes on at an equal rate at all points. Extension in the axial and circumferential directions are allometrically related, that is if C is the cell's circumference and H its length, then $dC/C = K\ dH/H$. K is twice as high at 36°C. as at 22°C.

Correlates of local growth.—The wall in expanding tips of the juvenile *Phycomyces* sporangiophore is much thinner than elsewhere (107). It may be, then, that here and in other cases of hyphal growth, the resulting higher tension in the wall's tip is in some manner responsible for the concentration of cytoplasm. Direct evidence that high wall tension can stimulate fibril synthesis is reported by Green (108). In *Nitella,* reduction of cell turgor, and hence wall tension, with polyethylene glycol solutions greatly inhibits cellulose synthesis.

Wilson's observations with visible light of the striations and scattering patterns on the walls of the largest cells of both *Valonia* and the related *Dictyosphaeria* (109, 110) when combined with the earlier x-ray work of Preston & Astbury (111, 112) and the electron microscope work of Steward & Mühlethaler (113, 114) yield a consistent and convincing picture: the cellulose fibrils in these cells lie in three systems of orientation, two spiral and one approximately meridional, which converge on their developmental poles.

One might imagine that the growth response of *Fucus* zygotes to polarized light is effected by the direct photochemical creation of an oriented molecular array which defines two developmental poles. However, the wave-length dependence and other details indicate that the phenomenon

is simply a variant of the directive influence of ordinary light on these zygotes, and more broadly of the general phenomenon of phototropism; specifically, it is suggested that the photoreceptor molecules tend to lie periclinally so that those molecules at the site of future rhizoid development are so oriented as to lie effectually in the dark [Jaffe (42)].

Lund (115) has measured the extracellular potential gradients on the surface of the filamentous green alga, *Pithophora* when the plant was suspended in moist air. The filament is septate with growth largely confined to the apex of the thallus. Within each cell cytoplasm including the chloroplasts accumlates apically and when branching occurs it originates from apices of intercalary cells. The potential distribution showed three main features: (*a*) Potential maxima (regions where the potential was most positive) were found at or slightly in back of the apex of each cell; (*b*) In moving basally from the apical cell, the height of successive maxima fell; (*c*) Potential maxima were also found near the cytoplasmic condensations associated with the laying down of a cross-wall during an intercalary division. These last two features of the distribution represent a correlation between positive extracellular potential and the site of wall growth. It would be of considerable interest to know if wall growth in *Pithophora* is concentrated at the apical end of each cell, as the distribution of cytoplasm and of branching suggests. For if it were, the positiveness of extracellular potential would be even more closely correlated with the rate of underlying wall growth, a conclusion on the cellular level which would conform to the correlation at a grosser level exhibited elsewhere (116 to 119).

If these potentials are like all other actively maintained biolectric potentials whose nature is at all understood, they will originate in the cell membrane. Hence the region just below the membrane which in turn underlies an extracellular locus of relaively high (i.e., positive) potential will be the site of a relatively negative potential as compared to other portions of the cytoplasm within this cell and should attract inclusions having a positive electrophoretic potential and repel those with a negative one. The available evidence, though sparse, consistently indicates that nuclei have a positive electrophoretic potential [Churney (120)], while chloroplasts have a negative one (121, 122). Put together, then, these facts suggest an electrophoretic explanation for the long established tendency of nuclei to migrate toward sites of wall growth in plant cells [Haberlandt (123)], and the sometimes associated displacement of chloroplasts away from these sites, as in normal (123) or regenerative (124) growth of *Vaucheria*.

Buller's (87) studies of hyphal fusion in a variety of Ascomycetes and Basidiomycetes are of fundamental interest. They strongly suggest that one of the links in the mechanism which originates and maintains tip growth is emission by the tip of an unstable substance(s) which in turn

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acts back on the emitting tip to speed its growth. In a tip-to-tip fusion, two hyphal tips grow close enough by chance (usually within about 10 to 15 μ). They then grow directly or almost directly toward each other, make contact and fuse. In peg-to-peg fusions, two older hyphae lying no more than 25μ apart mutually induce each other to form simultaneously peg pairs lying opposite each other which then grow toward each other, touch and fuse.

Whitaker put forward the plausible suggestion that the positive group effect in *Fucus*, i.e., the tendency of the eggs to germinate toward their neighbors, was simply mediated by a CO_2-pH gradient (33). However, a closer examination indicates that such a gradient does not in fact play a significant role in producing this interaction [Jaffe (125)].

Stadler (34) has studied a negative group effect in *Rhizopus*, a marked tendency of the conidiospores to germinate away from each other which is referred to as the staling reaction. He clearly confirmed the earlier demonstration of Clark that this reaction is due to a negative chemotropic response. He further shows that the grown mycelium produces an unstable substance which markedly inhibits spore germination (it could hardly have a half life more than about 30 min.), and makes the plausible assumption that this latter substance is the same as the one responsible for the group effect.

Heitz (49) reports that *Funaria* spores developing in diffuse white light tend to develop chloronemas away from their neighbors. However, since the chloronemas tend to grow toward unilateral white light, it is uncertain whether or not this negative group effect is simply a consequence of mutual shading.

Fischer & Werner's work (35) indicates that the tendency of hyphae of *Saprolegnia ferax* to grow away from each other is due, at least in part, to a positive chemotropic influence of amino acids in the medium. The chemotropic response is manifested both by rapid turning of the direction of growth of the hyphae and by the initiation of new laterals from the hyphal wall facing a source of amino acids. The remarkable discovery was made that the response cannot be elicited by any one amino acid; the simplest combination of acids giving more than a very weak response was leucine, plus glutamic acid, plus cysteine; various combinations of five or six amino acids gave somewhat greater responses; no one amino acid was essential to the response. In one experiment, swarmers germinating on otherwise nutrient-free agar were exposed to a gradient. From the data given, it can be estimated that in the threshold region the amino acid concentration lay between 10^{-5} and 10^{-4} *M* but the setup precludes any estimate of the minimal effective gradient.

Fischer & Werner hypothesize that the amino acids act to reduce the extensibility of the wall. However, in view of the fact, documented above, that in all cases investigated hyphae not bearing a terminal spore mass

extend through tip growth, and considering that the acids act to initiate new laterals toward the high amino acid side, there is hardly any doubt that in fact the amino acids act to increase the walls' extensibility.

Since antheridial hyphae of various members of the Saprolegniales were likewise attracted to an amino acid mixture, it has been suggested (126) that Raper's substance C (which attracts *Achlya* antheridia to the öogonia) consists of such a mixture. Whatever its nature, the action of substance C is remarkable with regard to the enormous distance, several thousand μ, from the öogonial source through which the 10μ wide antheridia can respond chemotropically (65). The so-called zygotropism of *Mucor,* etc., is likewise reported to appear at distances apart from the zygophores as great as about 2000 μ (66, 127, 128). But to my knowledge, there is no evidence that the initial long distance phase of this "attraction" does not in fact represent a self repulsion (via negative chemotropism) of the zygophores of the plus and also of the minus strain. Indeed, Banbury (127) presents some evidence that suggests, but unfortunately fails to prove, this point. Two other established cases of sexually significant positive chemotropism are the attraction of the trichogynes of *Ascobolus* [Bistis (94)] and of *Bombardia* [Zickler (129)] to the "male" cells; the maximal distances of attraction for *Ascobolus* are said to be about 20 μ [Raper (65)] and for *Bombardia,* judging from Zickler's photographs, about 50μ.

The work of Bopp (130, 131) extends the evidence of many predecessors that the moss calyptra quite generally exports a growth hormone to the developing sporophyte; for excision of the calyptra results in a more or less marked shortening and apical thickening of the developing sporophyte.

Bonner *et al.* (132), in a refinement of various older observations, report that the stipe of the commercial mushroom elongates primarily through the extension of axially oriented cells in a short region beneath the cap. The experiments of Urayama (133) furnish some evidence that the gills of this plant export a growth stimulator to these cells. Thus, if the gills are all cut off, agar blocks placed on the cut undersides of the cap and an excised gill placed upon one of these blocks, then the stipe bends away from this latter block. As much as 10^{-2} *M* indoleacetic acid was a completely ineffective substitute for the gill.

This last finding is in conformity with the emerging pattern of auxin action: it can only effect the elongation of cells with cellulose-bearing walls. (Basidiomycetes have chitinous, not cellulosic walls [Brian (134)].) Thus Davidson (135) made the striking observation that as little as 10^{-11} *M* indoleacetic acid markedly increased the length of the middle and inner walls of the green alga *Rhizoclonium hieroglyphicum* while not effecting any elongation of the outer wall; it is indirectly indicated by Smith (136) that the inner wall is cellulosic, the middle wall made of pectin, and the outer one chitinous.

Likewise, indoleacetic acid effects marked elongation of *Fucus* germlings at down to 10^{-8} *M* [Davidson (137)]; of *Nitella* plants regenerating from isolated internodal cells at down to 10^{-6} *M* [Sandan *et al.* (138)]; of apical segments of the green alga *Codium* at 10^{-5} *M* [Williams (139)]. According to the best available evidence, all these forms have cellulose in their walls. [For *Fucus,* see (25 p. 24), (140), (141); for *Nitella* and *Codium,* see (142).]

On the other hand, when indoleacetic acid is applied to a wide variety of fungi whose walls are not constructed of cellulose, no effects are observed except in concentrations $\geq 10^{-5}$ *M* (134, 143, 144).

Brian (134) has studied the effects of griseofulvin on 51 fungi including representatives of every major group. All 38 of the chitin-walled fungi showed distinct morphological responses to as little as 3×10^{-7} to 3×10^{-5} *M* solutions depending upon the species. On the other hand, all of the 12 fungi without chitinous walls showed no response to 6×10^{-5} *M* solutions. This latter group included 10 öomycetes and two yeasts.

The responses of *Botrytis alii* hyphae were studied most carefully. The lowest effective concentration, 3×10^{-7} *M,* causes "a marked spiral wave of the hyphae without noticeable reduction in the growth rate"; 3×10^{-6} *M* causes "excessive branching and distortion of the hyphae"; 3×10^{-5} *M* "causes the production of highly stunted and gnarled germ-tubes, usually with spatulate extremities," yet even 3×10^{-4} *M* neither blocks germination of the conidia nor causes any detectable change in O_2 uptake. Brian presents cogent arguments for the view that the griseofulvin acts by somehow producing a diffuse increase in wall plasticity. Thus the griseofulvin syndrome resembles that produced in root hairs by calcium deficiency, an effect which is apparently mediated by a reduced deposition of calcium pectate, the hairs' chief skeletal element [Cormack (145)]. Banbury (143) reports that 3×10^{-4} *M* griseofulvin in lanolin produces comparable reactions when locally applied to the *Phycomyces* sporangiophore. When applied unilaterally to the sub-sporangial growth zone in stage IV, the cell bends away from the griseofulvin, presumably because of greater wall growth there. It also tended to bend to the right as viewed from the griseofulvin and to continue growth in a clockwise helix as viewed from above. Similarly, application of the griseofulvin paste to the tips of sporangiophores before sporangium formation again yielded such clockwise spiraling. It should be noted that this griseofulvin-induced "spiraling" both here and in Brian's study is "helicogenic"—it produces a permanent helical form—and is geometrically distinct from the normal "spiraling" of the *Phycomyces* sporangiophore, a process which consists of a rotation of the growing wall about the longitudinal axis so as to lay down a straight sporangiophore [Castle (146)]. The griseofulvin-induced helicogenic spiraling appears to be a cogent argument in support of Frey-Wyssling's suggestion (147) that normal "spiral growth" of *Phycomyces* arises from a helically proceeding

path of localized wall growth. A large increase in the amount of local growth per unit path length in the postulated process would generate a visible and permanent helix instead of an apparently straight structure [See Castle's criticism (146)].

Protoplasmic movement.—Kühn (148) has clearly reviewed Steinecke's (149) important study of protoplasmic movements during the reversal of polarity in *Bryopsis*. In brief, there is apparently a movement of an apically situated "plasma" (as indicated by vital dyes) as well as apically aggregated chloroplasts to the former basal region and a concomitant movement of a "basal plasma" (again as indicated by vital dyes) to the former apex.

Köhler (150) has confirmed and extended older observations of the extreme tendency of the protoplasm in the cells of the filamentous green alga *Chaetomorpha* to flow basally. Swarmers germinating at an air-water interface develop an elongate tube instead of a basal holdfast; in each cell of an old filament transferred to a fresh medium the protoplasm shows a striking basal accumulation before dividing transversely. If an old filament is ripped off the substratum, it does not regenerate a holdfast but protoplasm from successively more apical cells forces its way to the basal tip, ballons out and dies.

Buller (87) has studied and reviewed protoplasmic streaming in the hyphal fungi. In general, flow is observed toward growing tips and also toward regions of relatively high external osmotic pressure. Buller believes that the regions evacuated by the flowing protoplasm are filled up largely by synthesis of new protoplasm on rich media and by vacuole growth on poor ones. Vacuoles are said to move with the stream only at relatively high streaming rates. It is necessary to explain the discrepancy between stream rates which are commonly of the order of 1000 μ/min., and tip growth rates which are of the order of 10 μ/min. Buller's report suggests that at least part of the difference lies in the fact that the rapid streaming is only observed in a few main hyphae; the total cross section of the growing tips may well greatly exceed that of the transport hypha which feeds them. Moreover, in the hyphae of the phycomycetes, though not in those of the higher fungi, a counterflow is clearly seen in the periphery of the hyphae.

The migration of the pseudoplasmodium of the remarkable new species *Dictyostelium polycephalum* [Raper (151)] provides at least a superficial transition from the intracellular protoplasmic movements considered above to the amoeboid movements to be considered next. Both myxamoeboid movements (151, 152) and hyphal tip growth (35, 102) commonly proceed at rates of the order of 10 μ/min.; the thickness of the *D. polycephalum* pseudoplasmodium (about 50 μ), is comparable to many hyphae; the slime sheath is at least superficially comparable to the hyphal wall.

It is in order to comment upon the intensively investigated phenomenon of aggregation in the cellular slime molds, a subject recently reviewed by

K. B. Raper (153). Except where counter indicated, this discussion refers to *D. discoideum*. In 1953, Raper reported that the process spreads out from collecting centers (19); in 1940 he reported that at low amoebal densities, amoebae reach the collecting centers from relatively distant points by means of conspicuous streams, rather than directly (154). In 1947, Bonner demonstrated that the initial stage in the aggregative movement was produced by positive chemotaxis (155). From these simple facts, it could have, but was not, deduced that a chemotactic chain mechanism is at work. That is, the chemotactic agent, acrasin, induces its own secretion, with a chain of such events starting at the primordial collecting centers. In 1953, Shaffer reported a critically important experiment which, among other things, made the above conclusion much more obvious, if not more compelling (156). Acrasin-sensitive amoebae were sandwiched between a block of agar and a supporting slide. The wash from centers was applied to the meniscus surrounding the block. Within 5 to 10 min., the amoebae began to move directly toward the nearest edge of the block and soon thereafter to form streams indicative of induced acrasin secretion (152, 156). Moreover, Shaffer pointed out that this chain mechanism is not only present, but that it increases the sizes of the aggregates far beyond the possibility of the direct mechanism (152).

The agar block experiments also demonstrated that acrasin in the wash from newly forming centers had a half life of the order of minutes. Obviously the more unstable a substance is, the more rapidly its concentration will decline on a trip from its source. Hence, Shaffer argues, an unstable chemo-orienting agent will be less likely to leave cells in the position of the ass between the hay stacks. Moreover, Shaffer presents a qualitative argument to show that as long as a source produces the chemotactic agent rapidly enough to maintain a concentration in the Weber-Fechner range, a cell can be lured to it over an indefinitely extended range by an unstable substance but not by a stable one (152). Additional conviction and clarity are lent by a mathematical argument. Routine solution of the diffusion equation for the case of a spherical source steadily emitting a substance suffering first order decay into an infinite medium yields the following simple result:

$$\frac{\Delta C}{C} = a\left(\frac{1}{r} + \sqrt{\frac{.7}{DT}}\right)$$

Where ΔC is the concentration difference across the test cell of diameter, a, of a substance with diffusion constant, D, and half life T, and at a distance r from the center of the source. (This expression never falls below $a\sqrt{.7/DT}$ which is 4 per cent where $a = 10\mu$, $T = 100$ seconds, $D = 5 \times 10^{-6}$ cm.^{2}/sec.)

An interesting delay was also observed in the agar block experiment. "A single addition of a concentrated acrasin solution (free of acrasinase)

produced dramatic orientation within 2-3 minutes" (152). It seems almost impossible to account for this lag on the basis of diffusion times. The dialyzable molecules of acrasin are very unlikely to have a diffusion constant, D, lower than 2×10^{-6} cm.²/sec. (157 pp. 13–14). For points close enough to the edge of the agar block and short enough times, the solution for the diffusion of a solute from an infinitely long column of solution into a similar column of solvent should be an adequate approximation. Using this model, one finds where $D = 2 \times 10^{-6}$ cm.²/sec., that a point 0.1 mm. from the block's edge will reach one-tenth maximal concentration in 26 sec. It seems a fairly safe conclusion, then, even from this preliminary report that there is normally a lag of at least a minute and probably more between chemotactic stimulus and response.

Now, Raper has found that *D. polycephalum* during aggregation sometimes exhibits conspicuous concentric zones of more and less concentrated amoebae; the wavelength being about .08 mm. [(151) Plate 2, Fig. 6]. It may be suggested that a stimulus-response lag plays a critical role in producing this phenomenon, as well as the "ripples . . . a few amoeba-lengths between their crests" reported by Shaffer (21) to appear in a film by Arndt of *D. mucoroides*. Each sparse zone would represent a region left behind by the group of stimulated amoeba proximal to it in the lag period before the stimulus was relayed from the proximal group to the next most distal group. The speed of chemotactic movement in the agar block test, which was of the order of 10 μ/min. or one amoeba length per minute is consistent with this interpretation (152).

Using the agar block experiment as a bioassay, both Shaffer (152, 158) and Sussman *et al.* (159) have presented strong evidence that the instability of acrasin is due to an enzyme. By various methods they inactivated and removed the putative enzyme and obtained stable crude concentrates of acrasin. Sussman, moreover, has presented preliminary evidence suggesting that acrasin consists of at least two components.

Bonner grafted the anterior section of a migrating pseudoplasmodium, labeled with a vital dye, on to the rear end of another intact pseudoplasmodium (160). The grafted front piece was then seen to migrate faster than the host so as to reach its front end within a few hours. Bonner puts forward the interesting suggestion that a process of velocity selection plays a part in determining the ultimate cell fate. In support of this, Bonner presents some evidence that even under normal circumstances, some of the individual cells move at rates different from the pseudoplasmodium as a whole. In assessing the significance of this process, one may note that of the 10 (?) members of the Acraseae carefully studied, only two, *D. discoideum* and *D. polycephalum* [Raper (151)], have a migratory phase, and even in *D. discoideum*, growth under relatively high temperatures or other slightly adverse conditions eliminates the migratory phase [Bonner (161); Raper (154)]. On the other hand, a process of velocity selection may well

play a general, if limited, role during aggregation. Thus, Shaffer (90) reports that nonaggregated myxamoebae show wide and persistent individual variations in their rates of turning in the absence of an acrasin gradient, and in their time of becoming reactive to acrasin gradients. Moreover, in aggregation streams, individual cells are sometimes seen to slip past each other.

Raper & Fennel (162) have published a study of "stalk formation in *Dictyostelium*," primarily *D. discoideum*. It includes a meticulous description of the process, an analysis of the sorophore sheath, and an analysis of the physical forces involved in the upward growth of the sorophore. It is argued that the two principal forces responsible for this process are "the swelling of stalk cells entrapped in the elongating sorophore sheath" and the surface tension of the sorogen, while a second and progressively diminishing force results from the coordinated pseudopodial movements of the myxamoebae that comprise the sorogen." Considering that the stalk cells swell to about five times their initial volume after deposition within the sorophore sheath, there can be no question that their swelling contributes a substantial fraction of the elongation of the cells after entrapment; however, that swelling and surface tension play a major, or indeed any, role in forcing cells into the sheath is open to serious doubt: (*a*) The only initial ascension rates published (those for fruiting bodies with relatively large sori) are those of Bonner *et al.* (163) and these vary from .8 to 1.3 mm./hr. These figures are little lower than the maximum rates of pseudoplasmodial migration found by Bonner *et al.* (164), namely 2 mm./hr.; the pseudoplasmodium, of course, is propelled solely by "pseudopodial movements." (*b*) It is true, as Raper & Fennel emphasize, that a diminishing fraction of the sorogen cells retain their power of amoeboid movement as ascension proceeds; it is likewise true that the rate of ascension diminishes during culmination (163). Moreover, it is notable that these cells which retain the power of locomotion always remain in a mass touching the sorophore sheath, which thus provides the necessary substratum for their traction. (c) It is true that the upper cells of the sorogen are oriented transversely to their axis of motion; but this is likewise true of the analogously situated anterior cells of the migrating pseudoplasmodium (161). (*d*) Recently Raper (153) has described the remarkable new species, *Acytostelium leptosomum*. In this form, the stalk is acellular.

Other polar gradients.—Heitz (49) reports that *Funaria* spores germinating under relatively dim white light sprout chloronemas first and rhizoids later or not at all. If, however, 6×10^{-11} *M* indoleacetic acid (or sometimes less) is added to the medium, then the spores sprout a rhizoid and no chloronema. This plainly suggests a significant role for auxin in determining the spore's polarity.

Various experiments suggest roles of indoleacetic acid in the establishment or maintenance of both the apicobasal and dorsiventral polarity of

the liverworts: extracted *Lunularia* tips yield an auxin as determined by the *Avena* curvature test, which a chromotographic study suggests consists solely of indoleacetic acid [La Rue & Narayanaswami (165)]. 10^{-5} *M* indoleacetic acid applied in an agar block to the cut apical tip of a *Lunularia* thallus whose apex was removed inhibits both apical regeneration and the germination of gemmae in the brood cups (165). As little as 6×10^{-9} *M* indoleacetic acid allows the dark germination of gemmae, which, because they developed on thalli in relatively little light, would not otherwise have germinated [Fitting (166)]. As little as 6×10^{-7} *M* indoleacetic acid weakens the directive influence of various external agents on the development of dorsiventral polarity in the gemmae of *Marchantia* [Kohlenbach (47)]. (See p. 364 of this review.)

Bonner (160) has vitally dyed vegetative amoebae of *D. discoideum* with neutral red, etc. With the otherwise undefined "beginning of differentiation" in the pseudoplasmodium, "the anterior region of presumptive stalk remains dark, while the posterior presumptive spore region in a matter of 10 to 15 min. bleaches considerably. The division line between these two regions is invariably clear and sharp." If one makes a "transection just at the division line between the bleached posterior section and the dark anterior section, then soon afterward the posterior end will again acquire a dark tip, and the anterior section will begin to blanch at its posterior end." It appears difficult to avoid the conclusion that the blanching and darkening represent processes of reduction and reoxidation.

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POSTHARVEST PHYSIOLOGY OF FRUITS[1,2]

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The last report covering the field of the postharvest physiology of fruits was published in this Review by Biale (1), following papers of Smock (2) and Miller (3). Nitsch (4) in his review on fruit growth discussed many problems of metabolism. Since that time much research has been done.

Some fruits after picking show important modifications characteristic of ripening, i.e., changes in colour, firmness, odour, flavour and composition, until the fruit becomes of "eating ripeness" in the case of edible fruits; others die without ripening. Thus the physiology of harvested fruits is chiefly the physiology of ripening and senescence.

Among the last general reviews dealing with this topic, we will mention Ulrich's books (5, 6). Much information may be found relating to the physiology of apples, lemons or cherries in the books of Smock & Neubert, (7), Bartholomew & Sinclair, (8) and Marshall (9).

We will review here successively the main new facts and opinions concerning the conditions of ripening, the gas exchanges, and the metabolism of postharvest fruits.[3]

THE CONDITIONS OF RIPENING

The nutritional and climatic conditions during the life of the plant have important effects on the postharvest behavior of fruits, but we need information on this point. Many physical or chemical agents may have significant effects on ripening of the fruit after picking.

Temperature.—Fruits picked at the right time generally can ripen at any temperature between two critical limits. For example, Williams pears ripen well between about 15°C. and 25°C. [or even at 1°C. according to Leonard *et al.* (10)]. Ripening failure of plums at 30°C. and 35°C. results, according to Claypool & Allen (11), from disturbances in the enzyme systems. Sometimes little differences in temperature may affect markedly the maturity (12).

It is curious that certain varieties which cannot ripen completely when put at 15 to 18°C. immediately after gathering need a cold treatment before

[1] The survey of literature pertaining to this review was concluded in March, 1957. Emphasis has been placed on literature published since 1950.

[2] The following abbreviations are used: DNP for dinitrophenol; DPN for diphosphopyridine nucleotide; DPNH for reduced diphosphopyridine nucleotide; 2,4-D for 2,4-dichlorophenoxyacetic acid; 2,4,5-T for 2,4,5-trichlorophenoxyacetic acid.

[3] We will suppose that the harvest is always made roughly at the end of the fruit growth.

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ripening at high temperature. For example, 11 to 15 weeks at 0°C. apparently represent good conditions for the Passe Crassane pear before ripening at 18°C.; ethylene has the same effect as cold pretreatment. The region in which the fruit was grown influences the need for cold [Ulrich *et al.* (13)]. Temperature does not affect the evolution of colour and firmness at the same rate [Leonard *et al.* (10)].

Radiations.—Radiations may act as stimulators or inhibitors of ripening. According to Ournac (14) grapes ripen more quickly under treatment with infrared radiations. Bananas irradiated with x-rays or other ionizing radiations exhibited a decrease in softening but an increase in skin blackening (15, 16).

Air humidity.—The relative humidity and the velocity of the air in the vicinity of the fruit influence the maturity, especially the evolution of the flavour. Saturated air hinders the development of a good flavour or odour in pears [Gac (17)]; apples may show blackening of the core under the same conditions (18).

Ethylene.—Ethylene is the most important of the chemicals used to effect ripening. Ethylene acts on the ripening of lemons, oranges, bananas, melons, and preclimacteric pears; the reaction is not so striking with peaches and apricots. Ethylene has usually no effect on the ripening of tomatoes [Fidler *et al.* (19)]. Smith (20) studied the effect of ethylene on plums and found that softening is more rapid than in air, but the taste remains unaltered. The case of the South Africa Kelsey plum is curious. In air the ripe fruit becomes yellow-orange; it can only turn purple after ethylene or acetylene treatment.

The effectiveness of ethylene treatment on ripening at ordinary temperature decreases when the preliminary cold storage period increases. During the last several years authors observed that the effects of ethylene on respiration and ripening are rather limited at low temperature; such conclusions are very important from a practical point of view (21, 22, 23).

Volatiles.—As Smock states, "probably no aspects of postharvest physiology of fruit is as controversial as that of the role of fruit volatiles" (24). Recent work by Maxie & Baker (25) suggests that nonethylenic volatiles can stimulate ripening. Smock's experiments (24) with air passing first over ripe apples and then over preclimacteric ones indicate that nonethylenic volatiles may influence the ripening of apples at storage temperatures. Air purification with activated carbon, sulfuric acid, and sodium hydroxide slowed down the ripening of preclimacteric apples in a recirculation system. Presumably autostimulation was checked by air purification.

However, Smock & Gross (26) observed a strange fact: the volatiles from large quantities of ripe apricots had a less stimulatory effect on the ripening of climateric apples than from smaller quantities at high temperatures. It seems that a depressing volatile is acting here; in fact activated carbon was able to remove this volatile.

The presence of ripe fruits under certain conditions has been found stimulating to unripe fruits. However, Gerhardt & Siegelman (*27*) have not been able to confirm this common opinion with apples and pears stored at −1°C. In the experiments of Fisher *et al.* (28), air purification had no influence on apples at −0.5°C.

Whether volatiles from ripe fruit can stimulate preclimacteric fruit in cold storage conditions is a problem still to be solved. Different conditions of temperature and differences in species and variety in experiments which have been made, explain the conflicting results of the authors. In cold rooms, nonethylenic volatiles may condense on the evaporator coils in considerable quantities (*29*).

Growth substances.—Growth substances are sometimes held to be stimulants of the ripening of gathered fruits; it seems that the treatment is only effective under certain conditions, particularly when application is made very early after picking. According to Freiberg (30), when stems of bananas were immersed in solutions containing 1000 p.p.m. sodium 2,4-D, 2,4,5-T, or para-chlorophenoxyacetic acid, ripening was accelerated when compared with controls. Attached fingers injected with 2,4-D turned yellow while adjacent untreated fingers were still green. Reducing sugar content in 2,4-D-treated bananas was higher than in controls. In the experiments of Stewart *et al.* (31), it was observed that 2,4,5-T especially and 2,4-D to a lesser extent when applied in a wax emulsion, delayed the development of yellow colour in the rind of lemons during storage. The storage life of the fruit was increased in these experiments.

It is clear that new observations are necessary in this subject.

GAS EXCHANGES

Pathways of Gas Exchange

After they have been gathered, fruits receive from the environment nothing but oxygen and sometimes water (vapour) or carbon dioxide; they give out at the same time carbon dioxide, ethylene, volatiles, and water vapour. The gas exchanges between the living cells of the fruit and the air are for the main part indirect because the internal atmosphere surrounding the cells has not the same composition as air. The circulation through the intercellular spaces, the lenticels, the skin, and sometimes through calyx, peduncle, and possibly superficial wounds is not always very easy.

The volume (e.g., 20 per cent of the total fruit volume in apples), the pressure (slightly lower than that of the external atmosphere) and the composition of the internal atmosphere of fruits have been studied, sometimes with original methods, by Hulme (32), Reeve (33), Smith (34), Marcellin (35), and Ulrich (36). The changes of the volume and composition of this atmosphere during ripening and senescence are important (*37*, 38, 39).

The structure of the fruit skin was investigated by Hall *et al.* (40); we

will discuss later the changes in the composition of its superficial coating. The number, structure, evolution, and efficiency of lenticels have been studied by Smith (34), Marcellin (41, 42) and Rosper (43).

The calyx is sometimes an important pathway for the circulation of gases (41). New methods for measuring permeability, using a circulation of air through the fruit, were thought out by Marcellin (44). Permeability of apples increases with the pressure of the air (Poiseuille's law), with the number of functional lenticels, or when large wounds are present, and decreases during ripening and senescence. It seems that only a little part of the water vapour and carbon dioxide evolves through the lenticels; an important fraction diffuses through the cuticle. Oxygen, on the contrary, which is less soluble in water and in lipides than carbon dioxide, enters through lenticels. A shortage of oxygen in the tissues may result (34, 40, 42). Pathways of gas circulation are not the same for different varieties and sometimes, in the same variety, they may differ from one fruit to another (i.e. the calyx may be open or closed). Many experiments have been made on waxing of the fruit surface to obtain a longer storage life (45, 46).

Respiratory Gas Exchanges

Methods.—Claypool and his co-workers (47) have shown in experiments on fruits that the rate of aeration can affect the carbon dioxide production. Two phenomena are involved: the retarding effect of carbon dioxide accumulation and/or oxygen depletion, and the stimulatory action of volatiles. Carbon dioxide output is not always a good test of fruit ripening. Thus, the rate of ripening of plums is not increased by high temperatures in the same proportion as CO_2 production (48); a peak of carbon dioxide may be observed without ripening in tomatoes (56). Concerning the physiological value of CO_2-output measurements or will mention Porritt's experiments (49). Addition of pyruvate or malate to apple slices produced an increase in CO_2 production but little or no change in O_2 uptake. Conclusions concerning the effect of acids on respiratory activity will be very different depending upon whether CO_2 production or O_2 uptake is used as a test.

The climacteric.—The general occurrence of the climateric rise has been recognized by Biale *et al.* (50); however, this phenomenon was not found by him in oranges and lemons which do not give off ethylene. Figs give no evidence of a climacteric peak (51) but cherries and apricots show one (52). When the climacteric does not appear in the Passe Crassane pear, ripening is also lacking or very slow (53).

Hulme (54) followed over a number of seasons the respiration rate of apples kept at 12°C. and 15°C. He remarks that there is a regular biennial variation in the level of respiration, and the difference in time between the onset of the climacteric at 12°C. and 15°C. varies with the date of picking

and thus might be used as a test of maturity. Moreover the ratio of rate of respiration to protein content at the climacteric peak (R/P) is surprisingly constant within one variety.

Eberhardt (55) has been able to obtain a typical climacteric not only with *Sambucus nigra* fruit but also with leaves of *Parthenocissus;* the respiratory activity becomes higher during the change of colour.

Sometimes the curves of respiratory activity do not show one but two or many maxima whose meaning is questionable (13, 56). We will discuss later the opinions given by different authors explaining the climacteric.

Effects of external conditions and of chemicals on respiratory activity.— The effect of temperature on respiration in apples was investigated by Rubin *et al.* (57); it is not the same for the skin and for the pulp and differs with the seasons. At low temperatures the CO_2/O_2 ratio of oranges increased; this observation led the authors [Metlitskii *et al.* (58)] to the conclusion that the respiration becomes more dependent on utilisation of organic acids. It was felt that this could explain the poorer taste of fruits stored at low temperature. Studies with stored cucumber fruits showed that at nonchilling temperatures (15°C.–30°C.) the rate of carbon dioxide production decreased with the duration of storage, whereas at chiiling temperatures (0°C. to −10°C.) the rate increased with time to a plateau that was then followed by a decline preceding death [Eaks & Morris (59)]. Carbon dioxide production by pears and apples placed in wet air or in air circulating at a high speed may be higher than that of fruits placed in opposite conditions [Gac (17)].

In the experiments of Claypool *et al.* on plums the increase in respiration was roughly proportional to the oxygen tension (11). The effect of oxygen concentration on the respiration of oranges has been investigated by Biale (60). It has been shown by Caldwell (61) that partial pressures of about 500 per cent oxygen have a specific effect of toxicity on apple tissues. Eaks *et al.* (62) found that during exposure at nonchilling temperature (15°C.) the respiration rate of cucumber fruits in air was a function of the oxygen concentration (1 to 16 per cent); however, at a chilling temperature (5°C.) oxygen concentration had little effect. Rubin *et al.* (64) state that the optimum in respiratory activity obtained for a certain concentration of oxygen is not the same for young and old apples or for 10°C. and 30°C.

In Fidler's experiments the respiration of apples was depressed in gas storage at 4°C.; a peak of respiration was observed which is similar to the peak of the climacteric rise in air; but Fidler believed that it was not the climacteric. The immediate effect of high concentration of carbon dioxide in the gas storage of apples is to induce a transitory rise of respiration followed by a decrease below the normal level (83).

It is well known that ripening and the climacteric can be prematurely induced at 20°C. by giving the fruit ethylene at low concentration, but it seems that at 3°C. the respiration is not sensitive to ethylene; at 7°C. the

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effect is slight but it becomes marked at 12°C. [Fidler (63)]. With apples stored at 5°C. in a continually renewed gas mixture, Griffiths & Potter (65) found that the continuous addition of ethylene (20 to 500 p.p.m.) had little effect upon the respiratory activity. Fidler (66) states that possibly the effect of ethylene on respiration is due to an uncoupling of phosphorylation, but the reason for this uncoupling is unknown.

Vapours given off by certain storage moulds of lemons (green mould) increase the respiration rate of these fruits; these moulds produce ethylene, but *Alternaria* which also stimulates the respiration of sound fruits does not give off ethylene [Biale (60)].

Postharvest applications of 2,4,5-T and ethylene at 21°C. accelerate the rate of respiration of preclimacteric pears. It is curious to notice that the effects of 2,4,5-T and ethylene were greatly reduced when applied to the fruit at 21°C. following a 15-day period of cold treatment at 2°C. [Uota & Dewey (67)]. In other experiments (68) the effect of 2,4-D on pomelos was to lower their respiratory activity and the level of the climacteric. A small but significant increase in respiratory rate of oranges and lemons was observed for both fruits in the presence of biphenyl vapour (69).

According to Todd (70) the respiration of mature picked lemons can be markedly stimulated by treatment with a mixture of ozone and 1-hexene at low concentration. Many authors have noticed that in the apple, the peel is the region showing the highest respiratory activity; this observation is perhaps correlated with the fact that the peel is the tissue where cell divisions continue longest.

Production of Nonethylenic Volatiles

Methods.—Important improvements have been obtained during recent years concerning the methods of investigation in this field. It must be noted that sometimes with too drastic treatments, decomposition products or derivatives may be obtained with the original volatiles. For collection of volatiles, three methods have been used: (*a*) distillation from fruit tissue or juice [White (71); Guadagni *et al.* (72)]; (*b*) adsorption on activated carbon from the air of a fruit store [Kuc *et al.* (73); Henze *et al.* (74)]; (*c*) collection of the products in reagent solutions or cold traps from air sent over fruits [Thompson (75); Meigh (76)]. It is possible to evaluate the total production of volatiles by complete oxidation of the products fixed by reagents or cold traps [Fidler (77)]. For identification of the different volatiles new methods have been investigated, including use of ultraviolet and infrared spectra and chromatography after preparing suitable derivatives. (76, 78, 79, 80, 81).

Total production of volatiles.—According to Fidler (66), the loss of carbon as volatile organic substances probably never exceeds 1 per cent of the loss as carbon dioxide. Cooking apples produce about 0.2 gm. of organic volatile substances per ton per day at 3°C., dessert apples about 3.5 gm. In

terms of carbon, ethylene forms 70 to 80 per cent of the total volatiles.

Mattus (82) stated that after cold storage, pears taken from a controlled atmosphere had lower rates of production of carbon dioxide, ethylene, and other volatiles than fruits taken from air storage. In the same way Fidler (83) found the rate of production of ethylene and nonethylenic volatiles from apples at 4°C. in air to be higher than in a gas storage [See also Porritt *et al.* (84)].

Turk & Van Doren (85) investigated the sorbates (alcohols, aldehydes, unsaturates) of activated carbon used for air purification in fruit stores. The relative prominence of these materials decreased markedly, with irregularities, as the season progressed, and a larger portion of the adsorbed materials consisted then of vapours of higher molecular weight and of aromatics whose source is unknown.

Gerhardt (86) made measurements of the rate of emanation of ethylene and other volatiles from pears and apples during storage at −1°C. and ripening at 18°C.; the nonethylenic volatiles evolved at a maximum rate of about 33 and 3.4 mg./kg./day for Bartlett and Anjou respectively and 6 to 24 mg. for different varieties of apples. The emanation increased with the progress of senescence; the maximum occurred with tissue breakdown and presence of decay organisms.

According to Uota and Dewey (67) the production of volatiles by pears is affected by ethylene and 2,4,5-T in about the same order as carbon dioxide production, but ethylene does not affect the volatile production when applied at 2°C.

Composition of the volatiles mixture.—We will only examine in some detail the results obtained with apples. The following components have been reported: (*a*) alcohols—methanol, ethanol, n- and isopropanol, n- and isobutanol, d-2-methyl-1-butanol, hexanol [White (71), Thompson (75), Henze *et al.* (87), Meigh (87a)]; (*b*) esters—esters of methanol, ethanol, 2-propanol, butanol, hexanol, and formic, acetic, propionic, butyric, caproic, caprylic, valeric acids (71, 75, 87); (*c*) aldehydes and ketones—acetaldehyde, 2-hexenal, caproaldehyde, acetone, n-butanal, propanal, ethylmethylketone, isobutanal, with probably isovaleraldehyde and methylpropyketone in traces and other carbonyl compounds (71, 74, 76, 78). Some of these substances are responsible for the odour and flavour of the ripe fruit.

Thompson & Huelin (88), investigating apples stored at 0°C. then removed from this temperature and put at 20°C. at intervals, found that in early removals, ester production at 20°C. increased to a maximum and then decreased; in later removals the increase was much less and finally became negligible. At 0°C. ester production increased steadily. Early picking reduced ester production and a higher rate of air flow increased it. Reducing the oxygen concentration to 6 per cent first increased and then decreased ester production in comparison with air.

According to Meigh (76), during storage, only two-carbon compounds

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were evolved in increasing quantities; the other volatiles decreased. There was no correlation in this experiment between a high rate of evolution of carbonyl compounds and heavy scald; rotting of the fruit did not increase significantly the evolution of carbonyl compounds. In pears [Luh *et al.* (89)] an excess of methanol and diacetyl might be used as a test of over-ripeness. Methanol of over-ripe pears might come from de-esterification of the pectin molecule by pectinesterase.

The volatiles produced by tomato, grapefruit, and grapes, were investigated respectively by Spencer & Stanley (90), Kirchner & Miller (91), Holley *et al.* (80).

Practical problems related to the production of volatiles.—The problem of the production of volatiles is very important from a practical point of view: it concerns the origin of scald and the effectiveness of air purification by activated carbon (92, 93) or by alkaline permanganate (94) in cold storage.

The scald causes serious trouble with apples stored at low temperatures. Numerous experiments have been made to understand the origin of the disease and the means to be used to avoid it (95, 96, 97). It appears from Smock's experiments that scald is inhibited when apples are dipped in a solution of diphenylamine after picking. The explanation of this protection is yet to be found; it may be due to inhibition of cytochrome oxidase (97, 98).

Some investigators (92, 99, 100) have observed a reduction in scald by means of purification of the storage atmosphere with activated carbon. Others (24, 66, 101) found no important reduction under the same conditions. Fidler (83), Thompson & Huelin (88) and Meigh (87a), suggest that volatiles play little or no role in the development of scald. After numerous experiments it seems incredible that volatile products should have no role to play in the production of scald. Recently Kuc *et al.* (73) undertook interesting investigations to determine which volatiles might be responsible for apple scald. Crude ether extracts from activated carbon used in commercial storage were shown to be very effective in producing scald-like injury. Esters comprised the most active fraction of the ether extract. Experiments with synthetic compounds confirmed these observations. The effectiveness of the active chemicals was not a simple function of their volatility or constitution but was also concerned with their capacity of penetration through the apple skin. Climatic conditions probably also play a role in the development of scald (102, 103).

Ethylene Production

Methods.—In the method of Stitt & Tomimatsu (104, 105) paper sensitized by red selenium to mercury vapour is used for estimation of ethylene, mercuric oxide being the oxidizing agent of this gas. Young *et al.* (106) published a manometric method for determination of low concentration of

ethylene which involved the production of a complex with mercuric perchlorate. Uota (107) also used the fixation on perchlorate but followed by oxidation with ceric salt. Stolwijk & Burg's method (108) is an absorption technique using mercuric perchlorate combined with the use of a katharometer; the study of ethylene production by tissue slices is possible in such conditions. In order to investigate very small quantities of ethylene, Phan Chon Tôn (109) adsorbs the perchlorate on silica gel. It will probably be possible to use gas phase chromatography successfully in the future.

Ethylene production.—A review on this subject was published by Porritt in 1951 (110). Hall (111), using a permanganate method, found that whole Valencia oranges produced considerably less ethylene than fruits cut into segments or inoculated with *Penicillium digitatum*. Many fruits from tropical and temperate climates were investigated by Biale *et al.* (50); species showing a marked climacteric give off ethylene, with the exception of the mango. The ratio of ethylene evolution to carbon dioxide output was highest for the apple, followed by the pear and peach. Orange and lemon do not exhibit any climacteric nor do they produce ethylene. The authors' opinion (50) is that ethylene is a product of the ripening process rather than a causal agent.

Uota's results (107) show that ethylene is produced by plums under conditions favourable to ripening. The variety Duarte produced practically no ethylene at 32°C. and did not ripen normally. Ethylene treatment prior to ripening increased the rate of ethylene production except at 2°C.

Pears and apples were investigated by Gerhardt (86). Bartlett pears produced a maximum of 0.87 mg. of ethylene per kg. per day after 84 days at −1°C.; this was more than 14 times the rate of the Anjou variety. The maximum emanation rate from apples during cold storage usually varied from 0.3 to 0.54 mg. Emanation was highest at 18°C.

The very young fruits (pears, cherries) seem not to give off ethylene; at the end of their growth, the quantity evolved is zero for cherries and 0.7 to 1.8 cc./kg./day for William pears, 2 cc./kg./day for Canada apples and 11 cc./kg./day for Reine des Reinettes apples [Phan Chon Tôn (112)].

According to Spencer (56), when tomato fruits were placed in oxygen, both ripening and evolution of ethylene were accelerated in proportion to the increase in respiration. Waves of ethylene evolution might or might not accompany the waves of CO_2 production.

The comparison of the emission curves of carbon dioxide and ethylene during ripening together with the fact that ethylene is not evolved under anaerobic conditions lead to the idea of a link between respiration and ethylene formation. However, at 40°C. [Hansen (113)] respiratory activity is high and ethylene production is minimum. On the other hand according to Stolwijk *et al.* (108), a concentration of DNP insufficient to cause a strong inhibition of respiration can stop ethylene production completely. *In vitro* studies using common compounds from plants and a crude enzyme obtained

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from apple juice or *Penicillium* revealed that ethylene arises by enzymic degradation of a number of active substrates, especially alcohol. In ripening fruit ethylene may act as an autocatalyst, accelerating its own production [Hall (111)].

Experiments on *Penicillium digitatum* suggest that ethylene could be formed easily from mannose, mannitol, critic acid [Fergus (114)], glucose, glycerol, and alanine [Phan Chon Tôn (115)].

In the experiments of Buhler *et al.* (116) carbon-labelled ethylene was introduced into the atmosphere surrounding fruits, but the uptake was small; ethylene is probably the terminal product of a chain of metabolic processes in fruit and cannot be further metabolized. The authors suggest that ethylene is probably synthesized by a pathway connected with organic acid metabolism.

Transpiration

Gac (17) paid attention to several problems relating to transpiration. He noted in particular that at low temperatures the transpiration intensity roughly corresponds to the loss of weight of the fruit. This relationship no longer exists at high humidities or when respiration becomes very active. A loss of water can be observed in saturated air because the fruit is then warmer than the atmosphere. Sometimes the external regions of apples stored in cold rooms become vitreous; apparently water distils from the center of the fruit to the surface. A minimum loss of weight takes place at the end of the preclimacteric stage. Water circulates through the tissues before being given off from the fruit mainly as liquid; evaporation takes place at the surface. The loss of water is a linear function of the deficit of humidity in the environment; the slope of the representative curve is proportional to the area of the fruit and to the velocity of the air.

Stewart *et al.* (117) and Freiberg (118) observed in lemons and bananas a significant reduction in water loss as a result of treatment with 2,4-D or 2,4,5-T. The treatment of citrus fruits with organic compounds of boron (albotenes) lowers not only the decay by mould development but also the loss of weight [Moreau (119)].

THE METABOLISM OF POSTHARVEST FRUITS

Oxidations and Respiration

Mechanism of oxidations.—The main substrates in respiration are sugars and organic acids. Fidler (120) using mature apples showed: (*a*) "that the loss of carbon as carbohydrate plus acid was equal to the production of carbon as carbon dioxide plus alcohol, in air and in nitrogen"; (*b*) "that the rate of loss of acid was unaffected by the presence or absence of oxygen"; (*c*) "that under anaerobic conditions the amount of carbon dioxide plus alcohol . . . was equivalent to the sum of the carbon dioxide and alcohol which would have resulted from fermentation of the carbohydrate

lost, plus the amount of carbon dioxide which could be produced from complete oxidation of the acid lost."

Oxidation-reduction potentials.—Oxidation-reduction potentials in fruit extracts were investigated in several papers (121, 122, 123). During the ripening period [Serini (124)] the potential is on the reduction side; while during over-ripeness, it is more on the oxidation side (pears, oranges, peaches).

Dehydrogenases.—The presence in protoplasmic particles of enzymes capable of oxidizing succinic, malic, citric, pyruvic, and fumaric acids was investigated by Millerd *et al.* (125) and Pearson & Robertson (126). More recently in experiments of Biale *et al.*, these particles showed high oxidative activity, particularly towards succinate and α-ketoglutarate. Adenylate was required for α-ketoglutarate oxidation. It appears from this that the particles (mitochondria) are organized enzyme complexes. They also contain cytochrome C and are able to oxidize reduced DPN and to reduce and oxidize cytochrome C. Therefore, the normal electron transport pathway may be as follows (127, 128):

$$\text{substrate} \xleftrightarrow[\text{DPN}]{} \text{DPNH} \xleftrightarrow[\text{Cyt C}]{} \text{reduced Cyt C} \longrightarrow O_2.$$

The presence of several dehydrogenases was also shown in the pineapple fruit (129).

In apples [Rubin & Gudalina (130)] the dehydrogenase system shows a maximum activity at about 37°C. to 40°C. Tests with methylene blue show that the dehydrogenase activity drops with ripening and aging of the fruit. In a later paper Rubin & Sal'Kova (131) showed the presence in apples of dehydrogenases effective on ethanol, malic, succinic, and isocitric acids. In green apples the malic dehydrogenase (investigated on tissue slices) is the most active. With ripening the activity of ethanol and malic acid dehydrogenases declines. In storage the process continues further. Succinic dehydrogenase shows a rise in activity up to harvesting, then shows a rapid decline. The various kinds of apples present considerable differences in dehydrogenase activities.

Oxidases and respiration.—The respiratory activity of young lemon fruit is inhibited by cyanide [Rubin *et al.* (132)] but not in old fruit; the cytochrome system would be active only in the case of young fruits. The same authors stated later (133) that at low temperatures apple respiration is carried on largely by polyphenoloxidase; cytochrome oxidase activity is higher in the fruit pulp than in the skin.

According to Markh & Feldman (134) the part of the respiratory activity catalyzed by metal enzymes decreases during ripening while the activity of the flavine enzymes increases at the same time. Polyphenoloxidase seems to be responsible for 50 per cent of the total respiratory activity. Webster (135) found that the respiration of apple slices in 95 per

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cent CO was inhibited in the same manner in light and darkness; probably cytochrome oxidase was absent. Despite certain contradictions, it appears from these and other experiments (128) that some enzymes different from cytochrome oxidase act in the fruit as terminal oxidation enzymes. At the last stage of ripeness lycopene could operate as a substitute of oxidase for carrying oxygen [Tombesi *et al.* (136)].

Oxidases and fruit browning.—Joslyn & Ponting (138) reviewed the problem and thought that in the protoplasm of an actively respiring cell, the oxidation-reduction potential is low enough to prevent the accumulation of oxidized phenols. In damaged tissues the phenol is probably oxidized faster or reduced slower than in the intact tissue. The browning seems to be due to the activity of polyphenoloxidase in the presence of *o*-diphenols and oxygen. In the living cells the phenols may not be able to react because of their location in vacuoles, while the oxidases are situated in the protoplasm. Biedermann (137) observed that the oxidase is strongly linked with the solid particles of apple or pear pulp; the enzyme is concentrated in the core and below the skin.

Bogdanski (139) investigated the browning of apple juices by measuring colour intensity and oxygen absorption of the liquid. Orthodiphenolase, peroxidase, catalase, and dehydrase are present. The author believed that with oxygen the phenol is oxidized by oxidase into quinone, the latter probably being reduced to phenol by ascorbic acid by the dehydrase. When all the ascorbic acid is oxidized, the phenols cannot reappear from quinones, and the juices become brown. It has, however, been found by Ingraham that catechol oxidation by polyphenoloxidase in air is not inhibited by ascorbic acid, but the dehydrase is lacking here (140).

Weurman and Swain (141) report that, although most scientists agree the enzyme system mainly responsible from browning is polyphenoloxidase, little information is available concerning the phenolic substrate. According to these authors chlorogenic acid takes part in the browning of apples and pears. As browning proceeds, chlorogenic acid decreases and three fluorescent compounds are formed from it during the reaction. Ascorbic acid and compounds containing —SH groups act as inhibitors; polyphenoloxidases from many sources are inactive below pH 4. However, Siegelman (142), using a paper chromatographic technique for the detection of the substrates of polyphenoloxidase, found in skin extracts of apples and pears l-epicatechol and in Bartlett pears smaller amounts of d-catechol. In spite of numerous attempts the problem of enzymic browning remains only partly solved.

Krebs' cycle and pentose cycle.—Experiments on tissue slices in the Warburg apparatus showed the stimulating effect on respiration of some organic acids (e.g., malic acid). The very low efficiency of citric acid in such experiments led to the hypothesis that the part of the Krebs' cycle involving this acid is slow or lacking in fruits. Tager's experiments on the effects of banana extracts on hexose-phosphates and ribose-5-phosphate

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and on the action on respiration of sodium fluoride indicate that the pentose cycle may be active in the respiration of preclimacteric bananas; near the climacteric rise, respiration via the Embden-Meyerhof-Parnas pathway seems to increase (143).

Respiration and phosphorylative coupling.—Throughout the period of ripening cytoplasmic particles prepared from avocado fruit showed a high ratio of phosphorus uptake per atom of oxygen used in respiration (125, 127).

The explanation of the climacteric rise.—It is generally believed that the respiration rate is governed by the amount of adenosinediphosphate which is available to accept phosphate. This in turn is dependent upon the rate at which adenosinetriphosphate is used in the various endergonic cell processes (synthesis). DNP is able to uncouple phosphorylation though allowing oxidation to proceed. With preclimacteric fruits the oxygen uptake becomes greater after addition of DNP, but after the climacteric rise oxygen uptake is only slightly affected by DNP [Pearson & Robertson (126, 144)].

Millerd *et al.* (125), using particle suspensions prepared from avocado tissues, observed the same relation, the phosphorylative coupling system seems to limit the respiratory rate in the preclimacteric fruit but not at the climacteric maximum. It was suggested that some material appears during ripening, which, like DNP, decreases the link between respiration and the phosphorylative systems. It is evident from these experiments that the relationship between oxidation and phosphorylation undergoes important changes in the course of the climacteric.

Eberhardt (55) noted that synthesis occurs in the fruit during ripening, including, for example, proteins, volatiles, and pigments; if ripening was marked by the uncoupling of respiration and phosphorylation, synthesis would not be possible.

In recent publications Hulme & Neal (145, 146) put forward new ideas on the origin of the climacteric of stored apples. They found that the addition of malate to peel discs from postclimacteric and senescent apples (but not from preclimacteric ones) causes a large increase of CO_2 output. This CO_2 appears through decarboxylation of malate in aerobic conditions and acetaldehyde is produced simultaneously; O_2-uptake did not vary at the same time ("malate-effect"). These observations are in accordance with the high respiratory quotients found during and after the climacteric. As acetaldehyde is toxic, a reaction other than conversion to alcohol must exist for its removal. The enzyme responsible for the decarboxylation of malate probably appears during the climacteric (judging from the protein accumulation observed by Hulme).

Sugars

Metabolism of polysaccharides.—Kidd *et al.* (147) investigated the degradation of starch in apples removed from the tree at different stages of

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development. After a short period during which synthesis of starch continues at a diminishing rate, degradation of starch seems to proceed at a rate proportional to the surface area of the starch grain. The synthesis of starch from carbohydrate decreases more rapidly at low temperatures than the process of starch degradation. The stopping of synthesis probably results from gathering, when translocation of precursors is no longer possible.

McArthur-Hespe, G. W. F. M., (148) investigating the activity of starch-splitting enzymes of pears found that the effect of α-amylase became greater at low temperature although starch was lacking. There is a certain parallel between the rise in α-amylase activity and the possibility of ripening after cold storage; if the rise in α-amylase activity is too great, ripening is made impossible. No important changes occur in the quantities of β-amylase during the storage period.

The cell wall material of Conference pears stored at 5°C. and 15°C. was investigated by Jermyn & Isherwood (149). The results show that the cell wall of the pear seems to be in dynamic equilibrium with cytoplasm, polysaccharides being both broken down and synthesized during ripening. A part of the carbon compounds of the cell wall becomes available for respiration. At 5°C. the depletion of the cell wall proceeds further than at 15°C. though at a slower rate. The labile material appears to be mainly araban. These conclusions agree with those from microscopic examination which shows that the cell walls become very thin and may collapse in over-ripe fruits. The highly lignified, thick-walled stone cells do not appear to alter during ripening.

Metabolism of soluble sugars.—A number of papers have been published on the chromatographic separation of soluble sugars in fruits (150 to 155); the presence of xylose is particularly striking.

Kidd *et al.* (156) investigated the variations in sucrose content of apples during storage at different temperatures and at different stages of development. Immediately after picking, sucrose (per apple) increases only a little; after that it decreases to a low level (logarithmic fall). Finally, it shows another lesser maximum at the time of the climacteric maximum; this second maximum is more striking at high temperatures (20°C.) than at low temperatures (1°C. or 3°C.). We also observed (155) in Williams pears an increase of the total soluble sugars and of fructose and sucrose during the ripening of fruits put at 15°C. immediately after picking. The changes in the amount of sucrose were very different according to the experimental conditions (cold pretreatment before ripening, temperature during ripening, etc.).

Some remarks may be added now concerning the pathways for the metabolism of soluble sugars. Transfructosidation is suggested by Ash & Reynolds to be active in the formation of ketose oligosaccharides; fructose might possibly come from sucrose (157). Axelrod & Seegmiller (158) observed after infiltration of radioactive glucose into apple discs, a formation of

radioactive sucrose (with most activity in the glucose part), but not of free fructose; the process was inhibited by exclusion of oxygen. Some activity appeared at the same time in amino acids (alanine, γ-aminobutyric acid, glutamine, asparagine). No evidence was obtained for a conversion of d-glucurono-γ-lactone to pentose by a decarboxylation mechanism in plum tissue [Hough & Pridham (159)].

Some papers have been published relating to derivatives of sugars, for example, alcohol, acetaldehyde and butylene glycol. Smith (160) investigated the alcohol and acetaldehyde accumulation in blackcurrant stored at 4.4°C. in air and in high concentrations of carbon dioxide (up to 50 per cent). This fruit can withstand a high concentration of alcohol without manifesting ill-effects; the low concentration of acetaldehyde was not sufficient to have a toxic effect; the ratio alcohol/aldehyde was, at the end of a four weeks' experiment, greater than 50.

In a series of papers Antoniani & Serini (161, 162) published the changes of the 2,3-butyleneglycol and acetylmethylcarbinol content of some fruits; they discovered a correlation between the content of these compounds and the stage of ripening of the fruit in apple, pear, *Cucumis melo* and *Diospyros Kaki.*

Pectic Components

In 1951 Kertesz published a very important book on pectic components (163). In the same year the publications of Maas (164) and Hottenroth (165) appeared.

Techniques.—Some new chemical methods have been published (166 to 171), but it is not certain that they always lead to the appreciation of the same substances. Therefore, it is difficult to compare several papers on this matter. It seems that many measurements are necessary to obtain a good idea of the pectic material of one fruit, for example, the amount of pectic acid, uronic acid, total acidity, methoxyl concentration, molecular weight, and others.

Metabolism.—Generally soluble pectin increases during ripening at the expense of protopectin [Roelofson (172)], but the qualitative changes of pectin are not well known. McCready & McComb state that the degree of esterification of the pectin with methyl ester groups was above 86 per cent in unripe peaches and pears; it dropped to below 40 per cent after ripening and the molecular size decreased (173).

Stier *et al.* (174) investigated the changes in pectin substances of red ripe tomatoes during a 21-day storage at 4.5°C. Protopectin decreased and pectin increased; this change was accompanied by a decrease in relative viscosity of pectin, pectate, and protopectin extracts (depolymerization). The methoxyl content of the fractions decreased in most cases during the storage period.

Fruit softening seems to be closely linked with respiration. Biale (175)

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noticed that in an atmosphere of 2.5 per cent oxygen, it took approximately two times longer for the fruit to soften than in air. Some common observations also seem to link respiration and fruit softening. Thus, the similitude of the curves of respiratory activity and pectin formation during the ripening, the stimulative effects on the two phenomena of temperature, ethylene and wounding, and the trouble observed simultaneously in pectic transformations and in respiration as effects of fruit coating all lead to the same conclusion (176).

Pectin can be broken down into galacturonic acid by ascorbic acid with or without addition of peroxides [Kertesz (163)]. According to Seegmiller *et al.* (177) there is no evidence that pectic substances are formed directly from galactose or galactan. In their experiments labelled glucose (^{14}C in the carbon C_1 position) was introduced into immature boysenberries. After two or three days galacturonic acid and arabinose were labelled. The degradation of galacturonic acid revealed more radioactivity in the C_1 position, which indicated that glucose was converted into galacturonic acid without cleavage of the carbon chain. Arabinose also seemed to be formed from glucose in a direct manner. In later experiments by Seegmiller *et al.* (178), glucose labelled with ^{14}C in the 1, 2 or 6 position and galactose 1-^{14}C were introduced into separate green strawberries through their stems. After several days the pectin was isolated and hydrolyzed. The results indicate that the intact hexose carbon skeleton is preserved in the transformation of glucose into galacturonic acid and arabinose.

Pectic enzymes.—[cf. review by Kertesz & McColloch (179)]. Two kinds of enzymes seem to be involved in the breakdown of pectic materials in ripening fruits: pectinesterase (P.E.) which splits the methyl ester linkages of pectins to yield pectic acids or pectate gels, and polygalacturonase (P.G.) which hydrolyzes the 1,4-α glycosidic bonds in the uronic chain to yield galacturonic acid. Pectolytic effects are also observed when certain moulds attack fruit tissues and during over-ripening of medlar fruits. McColloch *et al.* (180) found that both types of pectic enzymes occur in ripe tomato in greatest quantity near the surface of the fruit; the action of these enzymes is very rapid.

Pectinesterase.—Tomato fruits and, at a lower level citrus fruits, are good materials for the study of this enzyme. In Jacquin's experiments on apples and pears, the content of P.E. depended more on the maturity than on the variety of fruit. Pectin and enzyme increased during ripening (181, 182). The pectase activity in pears was investigated by Weurman by measuring the increase in viscosity of a solution of pear pectin added to enzymic extracts (183). Enzyme activity remained high up to the middle of July and declined afterwards to a low level two weeks before normal picking time. P.E. content of the picked fruit does not decrease as does that of fruit left on the tree; this observation suggests an explanation for some of the difficulties encountered with the ripening of pears after cold storage. The

peel of the ripe guava contains over three times as much P.E. as the pulp and seeds (184).

Polygalacturonase.—Weurman published several papers on pectinase of Doyenné Boussoch pears (185). He reports that the failure of earlier attempts to establish the presence of this enzyme in fruits is attributable to inhibitors. The P.G. activity was estimated by using its property to lower the viscosity of pectic acid solutions or by measuring the increase of reducing power of pectin solutions left in contact with the pulp. A thermolabile inhibitor, which can be isolated by precipitation with acetone, was found in the juice of three varieties of pears. The inhibition increases as the amount of inhibitor increases up to a maximum above which there is no further effect. At low pH values pectinase is more strongly adsorbed on cell material than the inhibitor.

McCready & McComb (173) could demonstrate no polygalacturonase activity in unripe fruits, but ripe pears and avocados showed some activity; the authors think that pectic enzymes come in closer contact with the pectic substances during ripening and hydrolyze them. According to Bell (186), pectolytic enzyme activity was weak in the immature fruit of *Cucumis sativus* but strong in the ripe fruit.

In 1955, McCready *et al.* (187) compared the action of tomato and avocado enzymes with that of fungal P.G. Both fruit enzymes act as do fungal enzymes and give galacturonic acid. It seems that only one kind of P.G. is present in the extracts. Enzymes develop during ripening and occur in measurable quantities only in ripe fruits.

In the experiments of Luh *et al.* (188) on tomatoes the products of P.G. activity were studied by paper chromatography. At the beginning a random cleavage of substrate was observed, and oligouronides were produced with the exception of free galacturonic acid; the reaction then slowed down continually, while galacturonic acid accumulated. In the studied varieties the P.E. activity was about 23 to 27 times as great as the P.G. activity (at pH 4.5).

However, the problem of pectin solubilization is not solved. Doesburg (189) wrote a short time ago that there seems to be no shortening of chains of pectin molecules during ripening. A lowering of Ca^{++} in the membranes accompanied by an increase of H^+ could explain solubilization.

Ascorbic Acid

In tissues, as in fruit juices, ascorbic acid may be oxidized by oxidases (phenolases, ascorbase) or dehydrases in the presence of quinones [Legrand & Bogdanski (190)]. Bogdańska & Bogdański (191) investigating apples, found after a four week period of storage at approximately 6°C. an average loss per day of 1 per cent from the initial amount of ascorbic acid, and after a further six week period of storage at an average temperature of 3.5°C., a synthesis or decomposition of l-ascorbic acid depending upon the

decrease of firmness of the parenchyma. Miller's investigations on physiological breakdown in pineapple (at low temperatures) show that the injured fruits had a very low content in ascorbic acid (192).

Some experiments must be reported concerning the synthesis of ascorbic acid. In the investigations of Fryer *et al.* (193) yellow-red tomatoes were ripened to the red ripe stage in a laboratory out of direct sunlight at 24°C.; the fruits elaborated ascorbic acid to a concentration which was approximately the same for red ripe greenhouse tomatoes. Mature green tomatoes in the same conditions, on the contrary, were not able to attain the normal content of the ripe fruit. The authors think that there is a critical stage of maturity between green and yellow-red at which tomatoes could be harvested without decrease of their potential ascorbic acid content (193).

Loewus *et al.* (194) injected detached ripening strawberries with glucose-1-^{14}C, glucose-2-^{14}C, galactose-1-^{14}C, sodium glucuronate-6-^{14}C, and glucuronolactone-6-^{14}C. The ascorbic acid was extracted and degraded for determination of the activities of the constituent carbon atoms. Ascorbic acid from berries treated with glucose-1-^{14}C contained 65 to 70 per cent of its activity in C_1, 14 to 19 per cent in C_6 and 7 to 18 per cent in C_3. Glucuronate-6-^{14}C and glucuronolactone-6-^{14}C did not give significant amounts of ^{14}C for ascorbic acid syntheses. It seems therefore that ascorbic acid takes its origin from glucose through a direct pathway without cleavage of its carbon chain, and not through glucuronolactone.

Organic Acids

General information concerning acidity of the fruit was published in this Review by Thimann & Bonner (195).

Acids found in fruit extracts.—Thanks to the new chromatographic methods in particular, numerous papers have been published dealing with the distribution and characterization of organic acids in fruits. We can mention only a few authors as examples. Reports on the following fruits include these for apple: Hulme [(196), chlorogenic acid (197), shikimic acid (198), citramalic acid (199, 200)], Buch [volatile acids (201)], Tanner & Rentschler (202); for pears: Anet & Reynolds [mucic acid (203)], Ash & Reynolds [galacturonic acid (204)], Bradfield *et al.* [chlorogenic acid (205)], Rentschler & Tanner [quinic acid (206)] and Ulrich & Thaler (155, 207); for plums: Dickinson & Gawler (208); for cherries: Thaler & Rateau (209); for peaches: Peynaud (210), Anet & Reynolds [mucic, quinic, and galacturonic acids (203, 211, 212)]; and for apricots: Anet & Reynolds (213). We have also to mention for keto-acids, the paper of Virtanen & Alifihan (214) and for volatile acids, that of Mehlitz & Matzik (215).

Quantitative fluctuations of the different acids.—According to Anet & Reynolds (212) the maximum of quinic acid occurred in peaches in the green fruits; on the contrary, citric acid increased at the end of ripening

and galacturonic acid was present only in ripe fruits. Hulme investigated the changes in α-ketoglutaric, pyruvic and oxalacetic acid content of apples during storage at 15°C. in nitrogen and after subsequent transfer to air; the three acids decrease in nitrogen and increase in air (196). Fidler (120) stated that the rate of loss of acid is proportional to the logarithm of the concentration and is constant for a given variety of apple. He found the surprising fact that the rate of acid loss is the same in anaerobiosis (in nitrogen) and in aerobic respiration.

Leonard (10) found that acids of Bartlett pears decrease during ripening except in the later stages. Ulrich & Thaler published the changes in the amount of different acids in the same variety; the maximum of quinic acid in the young fruit is the most striking fact (207). In clingstone cherries Serini (216) found no transformation of organic acids during cold storage (1°C.). According to Krotkov *et al.* (217) the pH of the juice of stored apples shows a slow and steady rise from harvest until the next spring. Several authors observed differences in the acidity of internal and external parts of the fruit; for example, Hulme found less quinic and shikimic acids in the pulp than in the peel of apples (198).

Pathways of synthesis and breakdown.—The relationship between acid and carbohydrate metabolism was discussed by Krotkov *et al.* (217). Organic acids do not contribute to the increase in sugars during the climacteric and are not formed during the decrease in sugars in the postclimacteric period.

Organic acid formation by carboxylation. Allentoff *et al.* (218) report that ripe McIntosh apples exposed for 18 hours in darkness to $^{14}CO_2$ fixed ^{14}C into malic, aspartic, and glutamic acids, α-alanine and serine. Malic acid and amino acids contained over 80 per cent of the ^{14}C incorporated in the nonvolatile compounds. Carbon dioxide fixation increases as the CO_2-concentration in the atmosphere is raised. Hulme observed that CO_2-injury of apples stored in an atmosphere containing 20 per cent CO_2 is accompanied by an increase in succinic acid content of the tissues. He suggested that this acid might kill the tissues (219). Ulrich & Landry (220), investigating Williams pears stored at O°C. in air enriched with 10 per cent CO_2, noted an accumulation of malic and succinic acids in the tissues. In the experiments of Buhler *et al.* (221) mature green tomato fruits were exposed in the dark to $^{14}CO_2$ for 48 hours; all the activity of both malic and citric acid was found in the carboxyl groups.

Hulme (200) studied the possible significance of citramalic acid in apples. He found citramalic and β-hydroxyglutaric acids yield acetoacetic acid by oxidation; acetoacetic acid could be an intermediate during oxidation of quinic and citric acids into malonic acid. Acetone which is given off by apples might originate from acetoacetic acid. It is possible that, *in vivo*, acetoacetic acid coming from fat metabolism gives rise to citramalic acid in the peel of the apple. After later experiments Hulme suggested the possibil-

ity of direct relations among succinate, malate, pyruvate, and acetaldehyde and between fumarate and succinate (146, 147).

Aromatic acids.—After having reported the isolation of l-quinic acid from young apples, Hulme (222) expressed his views on the biosynthesis of this acid. *In vitro,* quinic acid may lead to citric and aconitic acids by oxidation and to protocatechuic acid and shikimic acid. By combining with the relatively insoluble caffeic acid, quinic acid gives chlorogenic acid, also found in apples by Hulme (197). It is not certain, however, that shikimic acid comes from quinic acid *in vivo.* It has been suggested that quinic acid might provide a link between aliphatic and aromatic compounds in plants and that shikimic acid might be the first stage from quinic acid in the desaturation. The role of chlorogenic acid in fruit browning has already been mentioned above (141).

Amino Acids and Proteins

Amino acids found in fruit extracts.—Thanks to the chromatographic methods, a number of lists of amino acids present in fruits have been published. The methods have been reviewed recently by Hulme (224); the amino acids in fruits are reviewed by Joslyn & Stepka (225). We can mention in addition reports of only a few authors, e.g., for apples: Hulme *et al.* [methylproline and piperidine-2-carboxylic acid (226, 227) and other amino acids (228)]; Urbach [4-methylhydroxyproline (229)], McKee [many amino acids (230)]; for pears: Lawrence & Groves (171); for cherries: Thaler & Rateau (209); for oranges: Lansen & Jang (231), Townsley *et al.* (232) and Rockland & Underwood (233); for tomato: Andreotti (234); for banana: Hulme (235); for avocado fruit: Hulme [numerous amino acids (224)].

Changes of amino acids during ripening.—During the ripening of apricots, the amounts (percentage of dry matter) of aspartic and glutamic acids and asparagine decrease (236); during the same period the amounts of serine and valine increase steadily. These components arise from proteolytic processes.

Ulrich & Thaler (207) published observations on the free amino acids of William pears during ripening at 15°C. They found that lysine appears only at the end of the ripening and the increase of proline is the most striking fact during this period; aspartic and glutamic acids decrease progressively.

Pathways of metabolism.—Hulme (237) found a close parallel between the drift in the rate of respiration and in the protein N/non-protein N ratio in apples stored at 12°C. The fall in respiration occurring immediately after picking is accompanied by a concomitant fall in net protein in very young fruits in which cell division is taking place. Subsequently, in fruits of all ages when a climacteric rise occurs, it is accompanied by a striking increase in protein. Perhaps the new protein might contain enzymes active in respiration.

γ-Aminobutyric acid arises from decarboxylation of glutamic acid. Axel-

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rod *et al.* (238) investigated the glutamic decarboxylase in lemon's flavedo which is the richest object from this point of view. The amount of γ-aminobutyric acid could be used as a test of maturity for oranges.

Lipides

Interesting studies were made of the lipides from the peel of Granny Smith apples. Huelin & Gallop (239) isolated the epidermis of apple peelings and then extracted four fractions from it: oil, wax, ursolic acid, and "cutin." The oil fraction of the natural coating increased during storage to a maximum, three to four times the initial value; this increase was less important in gas storage. The oil content also increased with the maturity of the fruit at picking time. The iodine number of the oil increased with oil concentration. Small increases occurred in the wax, ursolic acid, and cutin fractions after prolonged storage. The fatty esters of the oil fraction were produced most rapidly at the beginning of storage. There was no correlation between oil content and resistance of the skin to gaseous diffusion although both increased during storage.

Davenport (240) published a more detailed analysis of the above-mentioned oil. This oil was extracted from mature apples which had been stored at 20°C. during six weeks in order to develop maximum oil content. Distillation was used for separation——stearic, arachidic acids were identified with smaller amounts of palmitic and behenic acids and acids of higher molecular weight (tetracosanoic, for example).

Carotenoids

Chromatographic methods are now often used in carotenoid investigations (241); paper chromatography was rarely applied to fruits (242).

Different pigments.—Goodwin (243) reported the results of a study on the distribution of polyenes in 23 species of berries; β-carotene seems to be always present. In the presence of lycopene β-carotene is often a minor constituent. When lycopene is present, one or more hydrocarbons of the phytofluene series is also present although tetrahydrolycopene is lacking. When β-carotene is the only hydrocarbon present, it is accompanied by xanthophylls and chlorophyll. Chlorophyll is not detectable in fully ripened lycopene-containing berries. Cryptoxanthin is almost always present. Lutein is often lacking. Xanthophylls in fruit differ from leaf xanthophylls by the fact that they occur mainly as esters [Karrer & Jucker (244)].

Among the authors who have separated the carotenoids from fruits, we will mention only studies on peaches, McCarty & Lesley (245); on oranges, Curl & Bailey (246); and on mangoes, Chaudhary (247). The different regions of the tomato fruit do not contain the same amount of pigments; according to McCollum (248) the outer pericarp is the richest in total carotenoids and the locular content is the richest in carotene at different stages of ripeness (fruits at 20°C.)

Effects of different factors on carotenoid content.—Nettles *et al.* (249)

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observed a better colour in tomato fruits picked at a green stage and ripened at 21°C. under light than in those ripened in the dark. Red light was particularly active. Better colour was obtained for fruits ripened at 16°C. than for fruits ripened at 4°C. McCollum (250) showed on the same species that in mature green fruits chlorophyll does not disappear and the carotenoids fail to develop in the absence of oxygen unless the fruits are illuminated.

According to Chaudhary (247) the development of pigments (xanthophyll and carotenes) in fully grown mangoes occurs in shorter time at 36°C. to 40°C. than at ordinary temperatures, but the maximum values are not changed. Exposure of the fruits to ultraviolet light increases the total carotenoid content during ripening.

Goodwin & Jamikorn (251) investigated the synthesis of polyenes in tomatoes during the ripening at 0°, 15°, 30°, and 37°C. At 37°C. lycopene synthesis was inhibited without an increase of phytofluene, ζ-carotene, or neurosporene content. Synthesis of α- and β-carotenes was very slightly inhibited at 37°C. At 0°C. all syntheses were inhibited. Hansen (252) stated that the storage temperature had no effect on the changes of colour of Anjou and Bosc pears.

Evolution of carotenoids during ripening.—Ripening is marked by an increase in the amount of carotenoids in the fruit. In tomatoes lycopene increased tenfold from the ninth to the twelfth day of ripening at 30°C. while phytofluene increased twofold [Goodwin & Jamikorn (251)]. Francis *et al.* (253) investigated the pigment changes in apples and found that they are mainly due to modifications in carotene content, rather than to variations in the amount of carotenols or chlorophyll.

After Goodwin's review (254) it seems possible that xanthophylls originate from hydrocarbons by oxidation and that β-methylcrotonaldehyde is the precursor of carotenoids; the different hydrocarbons may arise one out of the other or from a common precursor. The former opinion was brought out by Porter & Lincoln (255) after their interesting experiments on selection and genetics of tomatoes. Carotenes could arise from colourless polyenes by dehydrogenation, ring formation, and oxidation. Genevois' opinion (256) is that carotenoids might come from ethylene.

Phenolic Constituents[4]

This group of plant components is a very complex one, but our knowledge about them has increased during the last few years, particularly owing to the use of chromatographic methods. Among these components, flavones, anthocyanins and catechin derivatives have been recently investigated in fruits. Many papers concern the identification of the pigments: those by Siegelman (257), Masquelier & Point (258), Williams & Wender (259) for flavones; those of Sannié & Sauvain (260), Robinson & Smith [(261) straw-

[4] For chlorogenic acid, see organic acids above.

berry], Sondheimer & Karash [(262) strawberry], Bockian *et al.* [(263) grape], Ribereua-Gayon [(264) grape], and Dickinson & Gawler [(265) plum] for anthocyanims; those of Joslyn & Peterson for leucoanthocyanins in the pear (266); those of Johnson *et al.* (267), Williams (268), Rentschler & Tanner (269) for catechins and their derivatives.

Concerning the metabolism of phenolic compounds, one should mention that Pratt *et al.* (270) think that browning of stored strawberries may be due to interaction between anthocyanin and ascorbic acid or derivatives. Eberhardt (271) studied the relations between anthocyanin production and respiratory activity. Livingston & Markakis (272), after introducing ^{14}C into plants as $^{14}CO_2$, were able to isolate from strawberry fruit pelargonidin-3-glucoside ^{14}C.

Auxins

Nitsch (4) made an important report on the presence and role of auxins in the growing fruit. Only a few investigations have been undertaken with ripening and senescent fruits after harvest. Chromatographic methods are now generally used together with a biological *Avena*-test (273); however, Luckwill (274) used another test based on the parthenocarpy of tomato.

The fruit auxins are mainly localized in the seeds. Many promoting and inhibiting growth substances have been found in the seeds of apples (275), tomatoes (273), grapes (273), pears (276), cherries (277), black currants (278), beans (279) and strawberries (280). Three auxins have been identified: indole-3-acetic acid (strawberries, 280; cherries, 277; grapes, 273), indoleacetonitrile (tomato, 273; black currant, 278) and the ethyl ester of indoleacetic acid (apple, 281; perhaps grapes, 273). In coconut milk were found indoleacetic acid and other active compounds, in particular substances which induce cell division but no cell enlargement (282) and inhibitors.

One can demonstrate that the end of fruit growth and maturity are marked by a very low auxin content (274, 276, 277, 278, 283). During storage the auxin content remains very low and inhibitors are present (276, 277). If the auxins of the seeds are important to consider during fruit growth and in relation to fruit drop (274, 278), their study in postharvest fruit does not seem to be very useful. In any case it is probable that the state or the location of auxins in the cells is an outstanding factor. It would seem that the use of the *Avena* coleoptile as test material is not the most suitable for fruits.

CONCLUSIONS

Although numerous papers have been published since 1950, it is not yet possible to give a comprehensive view of the life of postharvest fruit. The gathering of the fruit certainly influences its physiology by stopping the arrival of nutrients; it probably hastens ripening. The evolution of the

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fruit is a consequence, first, of its initial structure and composition and second, of its metabolism, which is affected by the external conditions.

Many of the publications deal with the composition of the fruit or of fruit emanations (wax, organic acids, carotenoids, volatiles). The shortness of this review did not allow us to consider these biochemical problems. Among the specifically physiological investigations we have been able to discuss interesting ideas concerning phosphorylation coupling and oxidative properties of protoplasmic particles, decarboxylation and carboxylation in organic acid metabolism, the inefficiency of ethylene at low temperature, the existence of volatile inhibitors of ripening, inhibitors of pectic enzymes and skin evolution (lenticels, wax).

Important efforts have been made by the authors towards a better understanding of several physiological phenomena such as oxidations, climacteric, circulation of gases, action of pectic enzymes, metabolism of sugars, pectic components, and carotenoids.

Many improvements in techniques will help authors in the future, particularly these involving the use of chromatography, radioactive elements, spectrophotometry and as biological materials, cytoplasmic particles. Other possibilities are now available, e.g. the use of isolated fruits in sterile condition [Nitsch (284)], cuttings bearing fruits [Erickson & de Bach (285)] and fruit tissue cultures [Schroeder (286)].

The marked heterogeneity of fruits and the existence within them of gradients (287) led to the investigation of fruit slices or peel pieces for physiological research. This heterogeneity is perhaps related to the transport of certain components inside the fruit, or from the stalk to the berries [in reference to transport of minerals in grapes see Ournac (14)]. The use of tissue extracts or cytoplasmic particles is sometimes helpful because of the low permeability of whole cells to the chemicals used in the experiments. Unfortunately, the life of tissue or particle preparations is short and wounds may have important effects.

The end of life of a fruit is generally a slow agony, but it is often preceded by a period of great activity although growth is over. Ripening is characterized by intense oxidations, in which mitochondria play an important role; by the appearance or increase of certain constituents such as pigments, volatiles, ethylene, sucrose, soluble pectins; and finally, by the decrease of other constituents such as organic acids, chlorophyll and auxins. It is not possible to say if one of these modifications is the starting point of the whole series. It has been thought that ethylene production might play this important part, but it is now a very debatable opinion.

Efforts have been made to find some links between the different physiological reactions, for example between ethylene production and respiration, pectin evolution and respiration, between the different acids or carotenes, between acidity or ascorbic acid and pectin; but new experiments are necessary.

The ripening fruit is very sensitive to the external conditions (humidity,

injuries, composition of the atmosphere) and in particular to temperature, the action of which is more complex than would be a single stimulation of metabolism by heat (288).

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THE BIOGENESIS OF FLAVONOIDS[1]

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Introduction

The most recent review of flavonoid biogenesis was prepared by Geissman & Hinreiner and published in 1952 (1). Their review contains an excellent and thorough survey of naturally occurring flavonoids as well as discussions of the metabolic relations of flavonoids to other plant constituents. Earlier reviews of this subject include those by Blank (2) and Seshardi (3).

During the past few years, great advances have been made toward gaining an understanding of the relation of flavonoid biogenesis to other aspects of plant metabolism. Many of these advances are related to the theoretical contributions of Birch & Donovan (4). Some experiments suggested by their proposals have been performed and have proved to be very rewarding. The study of the relation of light to flavonoid biogenesis has also been approached with new ideas and new techniques recently. In addition, work of the past few years, largely through the insistence and example of Geissman and his co-workers, has shown the importance of examining the effects of each experimental treatment upon the accumulation of as many flavonoids and related compounds as possible in the same plant variety. This has been emphasized in genetics experiments.

The present review does not cover work on methods of analysis of flavonoids; this has been discussed very recently by Geissman (5). Also, observations on the occurrence of flavonoids not recognized at the time this subject was reviewed by Geissman & Hinreiner are generally not cited. The discussion of the chemistry of the flavonoids is also brief. Thus, this review is not an exhaustive report or analysis of the literature on all aspects of flavonoids and their biosynthesis; rather, an attempt is made to examine possible biosynthetic pathways and some physiological aspects of flavonoid biogenesis. The possible sequence of reactions in flavonoid biosynthesis is discussed in detail. The consideration of this portion of the problem is followed by a discussion of the role of light and certain other factors in flavonoid biosynthesis. The question of the function of flavonoids in plants,

[1] The survey of the literature pertaining to this review was concluded in November, 1957.

[2] The author wishes to express his appreciation to Drs. R. E. Alston, C. W. Hagen, Jr., S. B. Hendricks, H. Mohr, H. W. Siegelman, and K. V. Thimann for their generosity in making manuscripts available in advance of publication, and to Dr. E. D. Garber for helpful discussions of some segments of this review. The preparation of this manuscript was assisted in part by funds from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

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one of the most important problems in this field, is avoided in this review with the vigor which has characterized some other summary reports on flavonoids. Geissman (5) offers some interesting suggestions on the possible roles of these compounds.

Chemistry and Structure of the Flavonoids

The distribution of flavonoids and related compounds in the plant kingdom has been recently surveyed and is reported in the excellent review of Geissman & Hinreiner (1). Some of this material has also been included in Geissman's thorough review of methods for the analysis of flavonoids (5). For purposes of subsequent discussion, it will be profitable now to outline the structural relationships among the flavonoids. Recent work on the distribution and structure of leuco-anthocyanins will also be reviewed.

The flavonoid compounds can be characterized as C_6-C_3-C_6 compounds in which each C_6 portion is a benzene ring. The classification of flavonoids into major groups is based on the configuration and state of oxidation of the connecting C_3 portion of the molecule. Figure 1 (I-XI) illustrates these points. The conventions for numbering the positions in each category of flavonoids is also noted. The benzene ring to which the pyran ring (anthocyanidin) or the pyrone ring (flavone, etc.) is fused is designated the "A-ring" and the other benzene ring is the "B-ring."

The characterization of individual flavonoids within each major group is based on analyses of the pattern of substitution and on the nature of the substituents. Hydroxyl groups are the most common substituents and frequently one or more of them are utilized in the formation of ethers. Among the alcohols which participate in the formation of the ethers are methanol (methoxyl substituents resulting) and sugars. Glycosides of anthocyanidins are anthocyanins.

The substitution patterns found most commonly among the flavonoids are illustrated by the examples shown in Figure 2 (XII-XIX). Some generalizations regarding patterns of hydroxylation, methoxylation, and glycosylation from the review of Geissman & Hinreiner (1) are quoted below. (The numbering system used in the following summary refers to the fundamental nucleus XX.)

(*a*) The greatest number of compounds are hydroxylated at B-3,4, with B-4 and B-3,4,5 also common. Hydroxylation at B-2 is known, but no example is known of B-2,6 hydroxylation. (The term "hydroxylated" is intended to include the heterocyclic oxygen atom in the chromane or chromone ring of flavone derivatives . . .)

(*b*) Hydroxylation is always found at A-2, and in the great majority of cases at A-2,4,6. With a few exceptions, hydroxylation always occurs at least at both A-2 and A-4.

(*c*) Hydroxylation is known to occur at all possible positions in A; flavones are known which are A-2,3,4,5,6-hydroxylated.

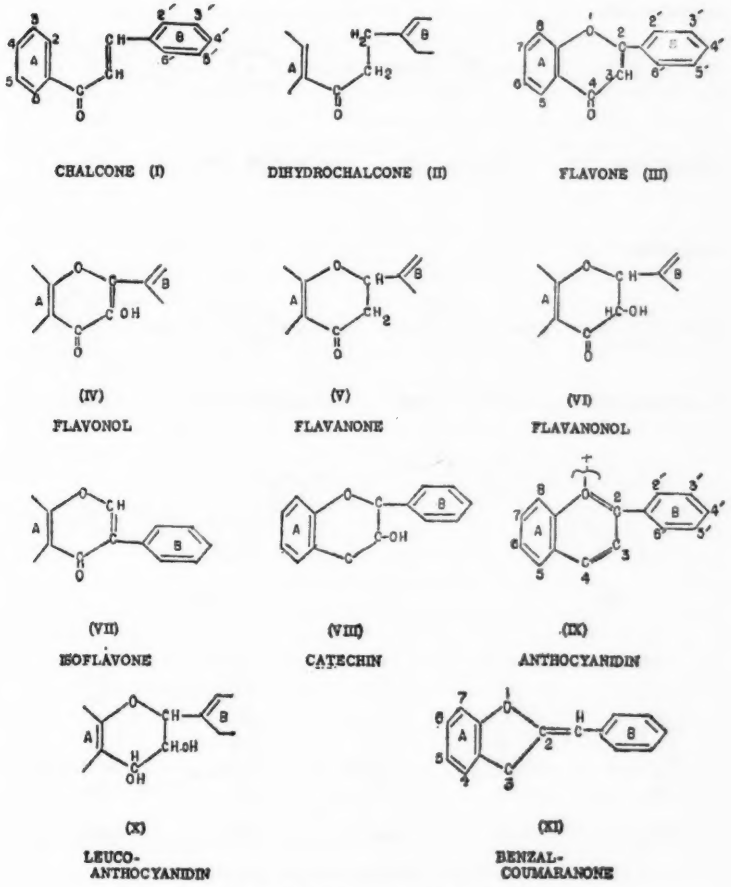

FIG. 1. Major groups of the flavonoids.

(d) The B-4 hydroxyl group in the anthocyanins is always free, never occurring in the glycosylated or methylated form.

(e) With the exception of the A-5 and B-2 positions, sugar residues have been found attached to any hydroxyl group in either ring, but are most commonly found at the A-4 and the C_3-β hydroxyl groups.

(f) Sugar residues are found only at the C_3-β or at both the C_5-α and the A-6 hydroxyl groups of the anthocyanins.

From an historical point of view, an important aspect of the types of

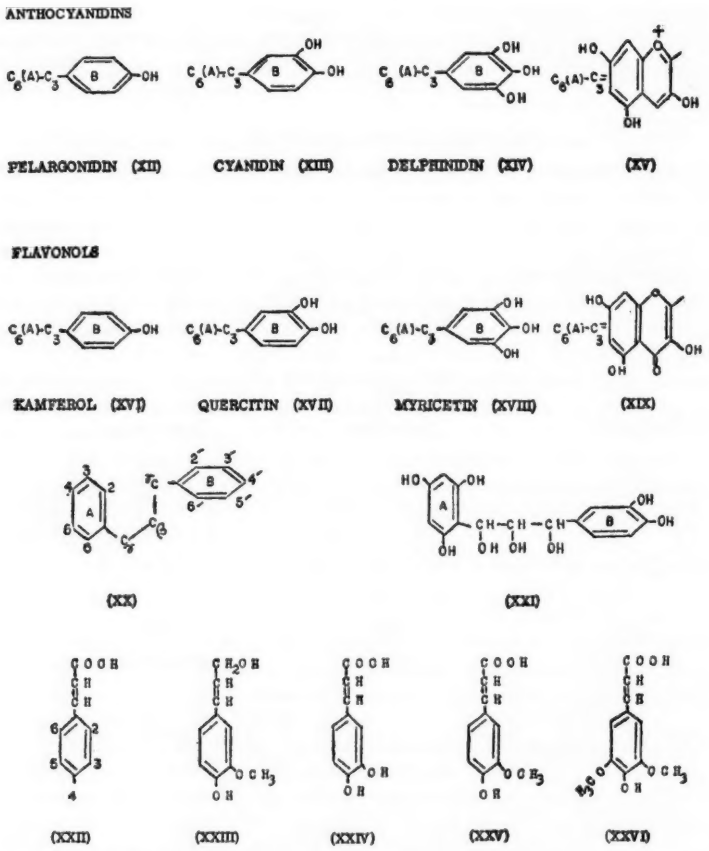

FIG. 2. Substitution patterns among the flavonoids.

hydroxylation patterns which are encountered is the common occurrence of a phloroglucinol arrangement of hydroxyl groups on the A-ring (e.g., pelargonidin, cyanidin, delphinidin) and of a tyrosine (e.g., pelargonidin, kamferol), catechol (e.g., cyanidin, quercitin), or pyrogallol (e.g., delphinidin) arrangement of hydroxyl substituents on the B-ring. These prominent features have been used as clues, reasonable though frequently misleading ones, to the course of biosynthesis of the flavonoids. Robinson (6) suggested that C_6-C_3-C_6 compounds are synthesized by the condensation of hexoses and trioses. This proposal was based largely on these observations: (*a*) Many hydroxyl groups occur on the anthocyanidin nucleus and the

state of oxidation of the anthocyanidins is generally close to that of carbohydrates. (Robinson points out that pelargonidin is at the oxidation level of a carbohydrate.); (*b*) anthocyanins accumulate at sites of intense photosynthetic activity when translocation of photosynthetic products is impeded, as in the case of injury, and (*c*) these pigments also accumulate when sugars are fed artificially, and in some cases of nitrogen deficiency. The hypothetical intermediate proposed by Robinson is shown in Figure 2 (XXI). This compound is at the oxidation level of the putative carbohydrate precursors. The Robinson hypothesis has had great influence on studies of flavonoid biosynthesis and was the basis for proposals that a $C_3(B)$-C_3 fragment, derived from carbohydrates, condenses with phloroglucinol to form the C_6-C_3-C_6 nucleus.

A great deal of interest has been focused on the nature and distribution of leuco-anthocyanidins during the past few years. Leuco-anthocyanidins were first observed by Rosenheim (*7*) in 1920 in acidic extracts of leaves of *Vitis vinifera*. He reported that conversion to anthocyanidins could occur in the absence of oxygen. G. M. & R. Robinson investigated leuco-anthocyanidins and suggested that they might be flavan-3:4-diols or 2:3:4-triols (8). The Robinsons isolated the leuco-anthocyanidin peltogynol from the heartwood of *Peltogyne porphyrocardia* (9). Mrs. Robinson demonstrated that an oxidation is required for the formation of cyanidin chloride from the leuco-cyanidin recovered from the gum of *Butea frondosa* (10). Bate-Smith (11) and Bate-Smith & Lerner (12) have reported the results of extensive surveys of leuco-anthocyanins among various groups of plants. They conclude that the presence, or absence, of leuco-anthocyanins in the leaves of vascular plants is related to the systematic position of the plants. These compounds are generally present in ferns, but have not been found in Selaginellaceae, Psilotaceae, Lycopodiaceae, or mosses. They are generally present in gymnosperms, but are absent from *Gnetum*. Their occurrence among monocotyledons is common but scattered. In the leaves of dicotyledonous plants, the presence, or absence, of leuco-anthocyanins appears to be associated with a woody habit in the plant, or in the forms closely related to it. Bate-Smith & Lerner suggest that "the production of leuco-anthocyanins is a part of a primitive metabolic pattern associated with, but not essential to, a tree-like or woody habit of growth."

Except in the Rosaceae and a few Leguminosae (which appear to contain leuco-peonidin), the leuco-anthocyanidins appear to be restricted to leuco-cyanidin and leuco-delphinidin. Leuco-pelargonidin has been reported to be present in the buds of *Impatiens balsamina* and the aleurone layer of corn kernels of the proper genetic constitution [Alston & Hagen (13,14); Coe (15)]. Finally, Bate-Smith & Lerner point out that the systematic distribution of leuco-anthocyanins "closely follows the recorded incidence of tannins in the botanical literature," and they suggest that the leuco-anthocyanins are probably the substances most commonly responsible for the re-

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actions in plant tissues attributed to tannins. A similar suggestion was made by Bate-Smith & Swain (16).

King & Bottomley isolated the leuco-anthocyanidin melacacidin from *Acacia melanoxylon;* this compound ultimately proved to be a flavan 3:4-diol (17, 18). King & Clark-Lewis (19) reduced the flavone corresponding to melacacidin with respect to its hydroxylation pattern, and isolated crystalline melacacidin from the reaction mixture; this provided conclusive evidence for the 3:4-diol structure (X). Bottomley described the conversion of melacacidin into 3,3′,4′,7,8-pentahydroxyflavylium chloride by refluxing the leuco-pigment in propanol-HCl, and suggested that this anthocyanidin had been overlooked in nature (20). It has now been found in the heartwood of *A. melanoxylon* by Hillis (21).

Bauer, Birch & Hillis (22) prepared synthetic leuco-anthocyanins by the reduction of flavonoids with lithium aluminum hydride in tetrahydrofuran. Similarly, Swain (23) prepared leuco-cyanidin by the reduction with sodium borohydride of the flavonone taxifolin isolated from the bark of the Douglas fir.

Keppler reports the isolation and crystallization of a leuco-anthocyanidin from *Acacia mollissima,* this compound is thought to be either 3′,4′,5- or 3′,4′,7′-trihydroxy-flavan-3:4-diol (24). Forsyth (25) described the production of catechins as well as cyanidin upon heating leuco-cyanidin from cacao beans in 0.1 *N* hydrochloric acid, but Keppler found no catechins after heating the *A. mollissima* leuco-anthocyanidin in acid solution. A leuco-anthocyanin or leuco-anthocyanidin which yields fisetinidin (3,3′,4′,7-tetrahydroxyflavylium chloride), as well as another leuco-anthocyanin which has not been identified, has been found in quebracho extract derived from the heartwood of *Schinopsis lorentzii* [Roux (26)].

Hillis (21) studied the distribution of leuco-anthocyanins in leaves, heartwood, sapwood, the cambial zone, and bark of various Eucalypts. He proposes that leuco-anthocyanins produced in the leaves may be the precursors of extractives in woody tissues. Among the many interesting observations made by Hillis were the following facts. The amount of leuco-anthocyanin present was inversely correlated with the degree of maturity of the different leaves on the stem, most of the leuco-anthocyanins appearing to be in the colorless leaf tips. The amount of leuco-anthocyanins in the bark decreased during the growing season, and the amount in the cambium decreased rapidly once differentiation of the tissues began. The leuco-anthocyanins present gave rise to cyanidin and delphinidin on heating in *iso*-propanol hydrochloric acid (the ratio of these two pigments varied from tissue to tissue and among leaves of different ages), while only delphinidin was identified in heated extracts of bark.

Kieser, Pollard & Williams (27) report that a major component of tannins of the Perry pear is a complex leuco-anthocyanin of high molecular weight composed of units similar in structure and hydroxylation pattern to cyanidin. The general problem of the relation of leuco-anthocyanins to

tannins and catechins will probably continue to receive considerable attention.

Some genetic studies on leuco-anthocyanins, as well as investigations of the possible role of these compounds as precursors of anthocyanins, are discussed in another section of this review.

The Formation of the C_6-C_3-C_6 Skeleton

General.—The relationships between flavonoid metabolism and some other routes in intermediary metabolism have become clearer in the past few years. The most direct links recognized at present are between flavonoids and acetate metabolism and flavonoids and other compounds with C_6-C_3 subunits.

The structural relationship of flavonoids to phenylpropane (C_6-C_3) derivatives of the sort found in essential oils, coumarins, lignans, and lignins has been recognized for some time. The usual pattern of hydroxylation (or other substitution) among the naturally occurring C_6-C_3 compounds, which include, e.g., cinnamic acid (XXII), coniferyl alcohol (XXIII), caffeic acid (XXIV), and ferulic acid (XXV), is that "a single hydroxyl substitutent almost always occupies the 4 position, while when two or more substituents are present the 3 and 4 positions are filled" (1). This has a strong resemblance to the situations found in the C_6(B)-ring of flavonoids and has called attention to possible metabolic relationships among these groups of compounds.

The study of lignin precursors and biosynthesis is linked closely to that of flavonoids. The results of some investigations of precursors of lignin are briefly summarized here. Wheat and maple plants supplied with C^{14}-labeled shikimic acid (Fig. 3, XXVII) produce radioactive lignin [Brown & Neish (28, 29)]. Also, lignin was isolated from sugar cane plants fed 2,6-C^{14}-shikimic acid. The vanillin which was prepared from this lignin was degraded and the labeling pattern in the aromatic ring was found to correspond to that of the shikimic acid which had been administered [Eberhardt & Schubert (30)]. Other compounds which have been shown to be efficient precursors of lignin in experiments with C^{14}-labeled compounds (29) are: phenylalanine, tyrosine, cinnamic acid (Fig. 2, XXII), and ferulic acid (Fig. 2, XXV). Schubert, Acerbo & Nord (31) have demonstrated that *p*-hydroxyphenylpyruvic acid-C^{14} is also utilized in lignin biosynthesis.

Thus, these data provide evidence that aromatic constituents of lignin are synthesized via shikimic acid and *p*-hydroxyphenylpyruvic acid. Dehydroshikimic acid, the immediate precursor of shikimic acid, appears to arise from 2-keto-3-deoxy-7-phospho-D-glucoheptonic acid which, in turn, is formed by the condensation of D-erythrose-4-phosphate with phosphoenolpyruvate (32, 33). The aromatic amino acids, which are also derived from shikimic acid (34, 35), are probably not on the direct synthetic path leading to lignins; there are marked species differences in the ability to utilize tyrosine for lignin synthesis (36).

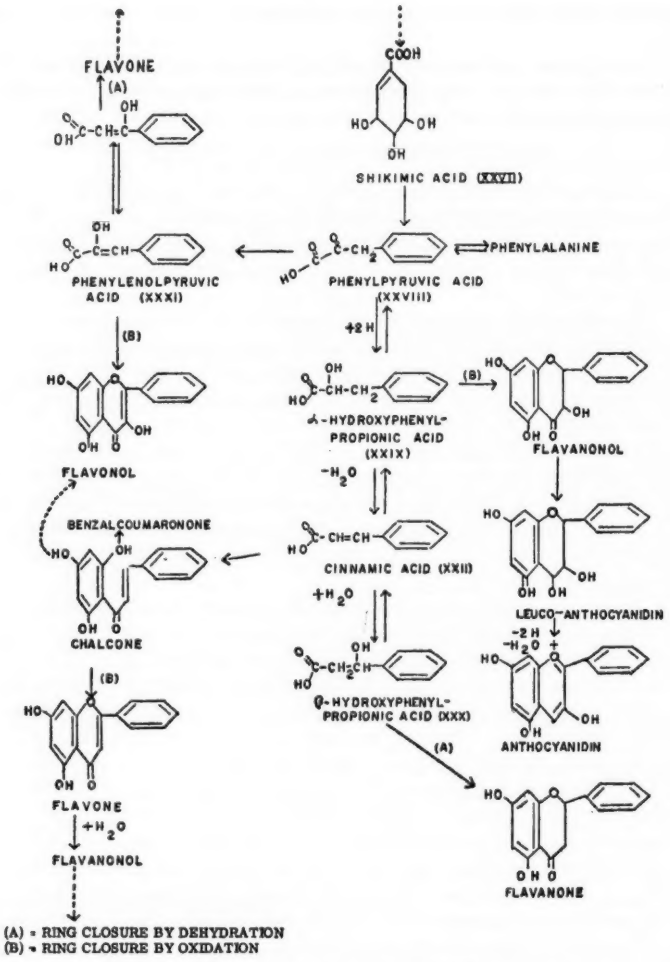

FIG. 3. Possible origins of various classes of flavonoids.

Origin of the $C_6(B)$-C_3 *unit.*—Shikimic acid has also been shown to be a precursor of quercitin (Fig. 2, XVII) in *Fagopyrum tatarcium* (buckwheat) by Underhill, Watkin & Neish (37). Buckwheat is reported to contain rutin, quercitin-3-rhamnoglucoside, at a concentration of 5 per cent of its dry weight and thus this plant appears to be particularly suitable for the study of flavonoid biosynthesis. In the experiments of Underhill *et al.*, buck-

wheat cuttings were supplied C^{14}-labeled substrate and maintained in the light for 24 hr. The quercitin was then isolated and degraded to phloroglucinol (containing all the carbons of ring A of the quercitin), which was found to contain 3.6 per cent of the radioactivity, and protocatechuic acid (containing all the carbons of ring B plus carbon 2), in which 93 per cent of the total radioactivity of the recovered quercitin was localized. Carbons 3 and 4 of quercitin are lost in this degradation procedure. Thus, shikimic acid is established as the precursor of ring B of quercitin.

Geissman and Harborne (38) have suggested that *p*-coumaric (4-hydroxycinnamic) and caffeic (3,4-dihydroxycinnamic) acids may be precursors of flavonoids. Presumably these acids are derived from shikimic acid. Flowers of normal snapdragon (*Antirrhinum majus*) contain apigenin (4′,5,7-trihydroxyflavone) and luteolin (3′,4′,5,7-tetrahydroxyflavone), but *p*-coumaric and caffeic acid esters accumulate in the flowers of the albino (-mm-nn) type which contains no C_6-C_3-C_6 pigments (38). These findings have been confirmed by Schutte, Langenbeck & Bohme (39).

Underhill, Watkin & Neish (37) have demonstrated the incorporation of radioactivity into quercitin (XVII) produced by buckwheat cuttings, supplied C^{14}-labeled caffeic acid (XXIV), *p*-coumaric acid, cinnamic acid (XXII), or any one of a number of other compounds. Their data indicate that all three carbons of the acrylic acid side chain of cinnamic acid are incorporated into quercitin without rearrangement. Radioactivity appeared almost exclusively in ring B of quercitin when ring-labeled cinnamic acid was administered. It is also of interest that all the carbons of phenylalanine are utilized, apparently without rearrangement, in the formation of quercitin.

Underhill *et al.* also estimated the relative effectiveness of the administered C^{14}-labeled compounds as precursors of quercitin in buckwheat by calculating the dilution of the radioactivity in the quercitin which was recovered; the dilution is equal to the specific activity of the compound administered divided by the specific activity of the quercitin recovered. Some of the compounds administered and the dilution values obtained are: shikimic acid, 36; L-phenylalanine, 66; cinnamic acid-B-C^{14}, 185; caffeic acid-B-C^{14}, 1268; sinapic acid-B-C^{14} (XXVI), 4240; and ferulic acid-B-C^{14} (XXV), 578,000. On the basis of these observations, Underhill *et al.* suggest that phenylpyruvic acid is the key C_6-C_3 intermediate in flavonol biogenesis. Phenylpyruvic acid (Fig. 3, XXVIII) has been shown to be an intermediate in the synthesis of phenylalanine from shikimic acid and it could also be derived from cinnamic acid with ease (Fig. 3).

Reznik & Urban (40) have shown that ferulic acid-B-C^{14} is incorporated into tricin (3′,5′-dimethoxy-4′,5,7-trihydroxyflavone) in wheat but no data are available on the effectiveness, quantitatively, of ferulic acid as a precursor. Reznik has advanced a scheme for the biosynthesis of the C_6(B)-C_3 segment of flavonoids; phenylalanine is an obligatory intermediate and caffeic acid is the key intermediate in this proposal (41). Geissman & Swain

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(42) have demonstrated that when phenylalanine-2-C^{14} is supplied to buckwheat (*Fagopyrum esculentum*) seedlings the quercitin and caffeic acid produced are both radioactive.

In summary: the $C_6(B)$-C_3 portions of quercitin, a flavonol, and of tricin, a flavone, have been shown to arise from shikimic acid or C_6-C_3 compounds whose origin can be traced to shikimic acid. It is reasonable to assume that shikimic acid is also a precursor of the $C_6(B)$-C_3 portions of other flavonoids.

There is no conclusive evidence to indicate whether hydroxylation of the $C_6(B)$-ring occurs prior or subsequent to the formation of the C_6-C_3-C_6 skeleton. Similarly, it is not clear whether there is with respect to the configuration of the C_3 side chain, a single C_6-C_3 compound which is utilized directly for the formation of all types of flavonoids, or whether the C_3 side chain is modified prior to the utilization of the C_3-C_6 compound for flavonoid synthesis. These questions will be discussed later.

Origin of the $C_6(A)$-ring.—Thus far, the origin of the $C_6(B)$-C_3 portion of flavonoids has been discussed. What of the other benzene ring? It is apparent from the data discussed above, that the A-ring does not arise from shikimic acid. It has also been shown that inositol, *meso*-inositol, and phloroglucinol, all frequently suggested as precursors of the A-ring, are not utilized for the synthesis of this ring [Watkin, Underhill & Neish (43); Weygand *et al.* (44)]. Experiments with *Chlamydomonas,* which indicate that phloroglucinol is an intermediate [Birch, Donovan & Moewus (45)], can apparently be disregarded [Ryan (46)].

An important theoretical contribution to the problem of flavonoid biosynthesis, and particularly to the question of the course of synthesis of the A-ring, was made by Birch & Donovan (4) in 1953. They suggested that particular flavonoids could be synthesized

> by the addition of three acetate units to cinnamic acid or related compounds. Such acids would presumably be related to phenylalanine, which is probably derived from shikimic acid . . . and hence from carbohydrate. Only the phloroglucinol or orcinol ring . . . would thus come from acetate units.

As the data discussed in the preceding section have shown, shikimic acid is indeed a precursor of the B-ring. The experiments cited below show clearly that the A-ring arises from acetate units.

Watkin, Underhill & Neish (43) found that C^{14}-labeled quercitin (XVII) was produced in buckwheat cuttings supplied with C^{14}-labeled glycerol, glucose, sucrose, or sodium acetate. In these experiments, quercitin was isolated and the pentaacetate derivative was prepared and degraded to phloroglucinol tetraacetate (which contains the carbons of ring B plus carbon 2). Quercitin, recovered from buckwheat cuttings supplied with C^{14}-sucrose, was almost uniformly labeled while radioactivity was almost entirely confined to the A-ring of the flavonol when either methyl- or carboxyl-labeled acetate were fed. No data are given for the distribution of

C^{14} in quercitin from plants supplied glycerol-1,3-C^{14}. (The participation of phenylalanine in the formation of the $C_6(B)$-C_3 portion of quercitin is also supported in an experiment in this series. When $C^{14}O_2$ and non-radioactive phenylalanine were supplied, almost all of the radioactivity of the quercitin produced was found in ring A, but when the phenylalanine was omitted, uniformly labeled quercitin was recovered.) Geissman & Swain (42) have demonstrated that buckwheat (*F. esculentum*) produces quercitin-C^{14} when supplied acetate-2-C^{14}. The appearance of radioactivity in the B-ring of quercitin and in caffeic acid indicates that more mixing of components of metabolic pools occurred in these experiments than in those of Watkin *et al.*

Grisebach, almost simultaneously with Watkin *et al.*, reported on the distribution of C^{14} in cyanidin prepared by the hydrolysis of rubrobrassicin synthesized in red cabbage seedlings which had been supplied methyl- or carboxyl-labeled acetate (47). Grisebach carried the degradation of the flavonoid farther than did Watkin *et al.* (43), so that the radioactivity in carbons 1, 3, and 5 could be measured separately from that of carbons 2, 4, and 6 (numbering as in XX). When carboxyl-C^{14}-acetate was supplied, carbons 2, 4, and 6, the hydroxyl-bearing carbons of the A-ring of cyanidin, were found to contain 89 per cent of the activity of ring A. Thus, in two cases, the synthesis of cyanidin (XIII) and of quercitin (XVII), it is clearly established that ring A is synthesized from acetate units in accordance with the suggestions of Birch & Donovan (4). Furthermore, the labeling pattern shows that the acetate units are added to the $C_6(B)$-C_3 segment either by the successive addition of three acetate units, or, possibly, by the addition of a preformed triacetate derivative of the kind described by Witter, Stotz and their co-workers (48 to 51).

The initial condensation might well be of $C_6(B)$-C_3-Coenzyme A (e.g., perhaps, phenylpropionyl-Coenzyme A, cinnamyl-Coenzyme A, or phenylpyruvyl-Coenzyme A) with acetyl-Coenzyme A. Suceeding additions of acetate units could occur by reactions analogous to some of those involved in the synthesis of fatty acids (52). Suggestions of this sort have been made by Birch & Donvan (4) and by Watkin *et al.* (43).

The minimum sequence of reactions required to fit this proposal appears to be as follows:

(A) Co ASH + ATP + C_3-$C_6(B)$ → Co A-S-C_3-$C_6(B)$ + ADP + P_i
(B) Co ASH + ATP + COOH-CH_3 → Co A-S-OC-CH_3 + ADP + P_i

(C) Co A-S-C_3-$C_6(B)$ + Co A-S-OC-CH_3 → Co A-S-OC-CH_2-C_3-$C_6(B)$ + Co ASH
(D) Co A-S-OC-CH_2-C_3-$C_6(B)$ + Co A-S-OC-CH_3 →
Co A-S-OC-CH_2-CO-CH_2-C_3-$C_6(B)$ + Co ASH
(E) Co A-S-OC-CH_2-CO-CH_2-C_3-$C_6(B)$ + Co A-S-OC-CH_3 →
Co A-S-OC-CH_2-CO-CH_2-CO-CH_2-C_3-$C_6(B)$ + Co ASH
(F) Co A-S-OC-CH_2-CO-CH_2-CO-CH_2-C_3-$C_6(B)$ →
Co ASH + COH=CH-COH=CH-COH=C-C_3-$C_6(B)$

C_3-$C_6(B)$ + 4 ATP + 3 COOH-CH_3 → $C_6(A)$-C_3-$C_6(B)$ + 4 ADP + 4 P_i

The nature of the $C_6(B)$-C_3 reactant is not specified. Birch & Donovan (4) suggest that the cyclization of the six carbon progenitor of the A-ring might proceed by aldol condensation or C-acylation [reaction (F) above].

The reductive steps which occur in the formation of fatty acids are omitted in the "minimum sequence" required for the synthesis of the $C_6(A)$-C_3-$C_6(B)$ skeleton. Steps (G), (H), and (I) would fit between reactions (D) and (E) and an analogous sequence would occur between (E) and (F) if the reductive reactions of the fatty acid cycle were added to the "minimum sequence" for flavonoid synthesis.

(G) Co A-S-OC-CH_2-CO-CH_2-C_3-C_6(B) + 2H → Co A-S-OC-CH_2-CHOH-CH_2-C_3-C_6(B)
(H) Co A-S-OC-CH_2-CHOH-CH_2-C_3-C_6(B) →
Co A-S-OC-CH_2-CH=CH-C_3-C_6(B) + H_2O
(I) Co A-S-OC-CH_2-CH=CH-C_3-C_6(B) + 2H →
Co A-S-OC-CH_2-CH_2-CH_2-C_3-C_6(B)

If reactions (G), (H), and (I) are included in the synthesis of the $C_6(A)$-C_3-$C_6(B)$ skeleton, oxidations will be necessary to aromatize and hydroxylate the A-ring.

Modifications of the C_6-C_3-C_6 Skeleton

Hydroxylation of the B-ring.—The experiments described in the preceding sections have provided evidence which clearly establishes the origins of the carbon skeleton of the flavonoids and, by analogy with other biochemical systems (e.g., the synthesis of fatty acids), the course of assembly of the smaller preformed units into a $C_6(A)$-C_3-$C_6(B)$ compound is inferred. One of the unresolved major questions in flavonoid biosynthesis is this: Does hydroxylation or modification of the hydroxylation pattern of the B-ring occur prior to the addition of acetate units to the $C_6(B)$-C_3 fragment or subsequent to the formation of the C_6-C_3-C_6 skeleton? If the former is the case, there should exist interconvertible pools, or at least mechanisms for interconversion, of 4-hydroxy-, 3,4-dihydroxy-, and 3,4,5-trihydroxy-$C_6(B)$-C_3 compounds. If changes in the hydroxylation of the B-ring occur after the formation of the flavonoid, there should be, with respect to the hydroxylation of the benzene ring, some single precursor [e.g., 3,4-dihydroxy-$C_6(B)$-C_3]. Pertinent unequivocal evidence, which could facilitate a choice among the alternatives presented above, is not available. Existing evidence comes from biochemical and genetic investigations.

Studies on the biosynthesis of lignin have shown that mechanisms for the hydroxylation, as well as the methoxylation, of the 3, 4, and 5 positions of C_6-C_3 compounds exist in plants. For example, hardwood lignins contain 3,4,5-hydroxy- or methoxy-substituted cinnamic acid derivatives and yet phenylalanine can serve as a precursor of lignin in maple [Brown & Neish (28, 29)]. Evidence has also been obtained which suggests that each type of lignin polymer has a specific aromatic monomeric precursor with a cor-

responding pattern of hydroxylation and methoxylation. Thus, sinapic acid appears to be preferentially transformed into syringyl lignin, ferulic acid into guaiacyl lignin, and probably *p*-hydroxycinnamic acid into *p*-hydroxy lignin. Direct evidence for the hydroxylation of the aromatic ring of a C_6-C_3 compound has been obtained by Geissman & Swain (42). They provided seedlings of *F. esculentum* and *Nicotiana tabacum* with phenylalanine-2-C^{14} and subsequently isolated radioactive caffeic acid. These experiments show that the hydroxylation pattern of the B-ring could be determined prior to the completion of the C_6-C_3-C_6 skeleton. It appears reasonable to expect that the enzymes which catalyze the hydroxylation of C_6-C_3 precursors of lignin could serve as well for similar precursors of flavonoids. Do the results of other investigations support the view that the hydroxylation pattern of the B-ring is determined early in the biosynthesis path which leads to flavonoids?

An extensive analysis of the large body of information on the inheritance of flavonoids is beyond the scope of this review but work in genetics has shown that, usually but not always, the direction of mutation from the wild-type is toward pigments with fewer hydroxyl substituents on the B-ring (Beale, 53), and that more extensive hydroxylation of this ring is dominant to the less hydroxylated state [see reviews by Scott-Moncrieff (54) and by Lawrence (55) for work to 1950]. Most of the genetic evidence is not inconsistent with the view that the hydroxylation pattern of the B-ring is established prior to the formation of the flavonoid, but the results of many of these experiments can also be interpreted as being not inconsistent with the opposite view. These are a few examples: In *Streptocarpus,* the 4′-hydroxyanthocyanin,[3] pelargonidin-3-pentose-glycoside, is found in flowers of plants with the genetic constitution *ord;* the 3′-methoxy-4′-hydroxyanthocyanin, peonidin-3-pentose-glycoside, in plants of genotype *Ord;* and the 3′,5′-dimethoxy-4′-hydroxyanthocyanin, malvidin-3-pentose-glycoside, in flowers of genotypes *Ord* or *ORd.* In *Lathyrus odoratus,* plants of genotype *ee smsm* contain pelargonidin derivatives (4′-hydroxy); plants of genotype *ee Sm-* contain cyanidin derivatives (3′,4′-dihydroxy); and plants of genotype *E- Sm-* contain delphinidin derivatives (3′,4′,5-trihydroxy) (54). In *A. majus,* Geissman and his co-workers (56, 57) report that flowers of plants with gene *M* contain glycosides of the 3′,4′-dihydroxyflavonoids, quercitin, luteolin, and cyanidin, and the 4′-hydroxyflavone, apigenin; in the absence of *M,* that is, in flowers of plants of the genetic constitution *mm,* only glycosides of 4′-hydroxyflavones (apigenin, pelargonidin, and kamferol) are present. Dayton (58) reports that at least one glycoside of apigenin was observed in flowers of all *A. majus* varieties she examined except the dead-white "yy-type." Flowers of *bb* plants produce

[3] The substituents on only the B-ring are noted in the discussions in this section, e.g., pelargonidin, 3,4′,5,7-tetrahydroxyflavylium chloride, is referred to as "pelargonidin (4′-hydroxy)."

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pelargonidin (4′-hydroxy) derivatives and two apigenin glycosides which occur only in the presence of pelargonidin; flowers of *B-* plants contain no pelargonidin derivatives but instead accumulate cyanidin (3′,4′-dihydroxy) glycosides sometimes accompanied by luteolin (3′,4′-dihydroxyflavonol). Luteolin occurs only in the presence of gene *B* in this material. Geissman *et al.* report that, in *Dianthus caryophyllus* (carnation), flowers of *rr* plants contain glycosides of the 4-hydroxyflavonoids, kamferol and pelargonidin, while flowers of *R-* plants lack pelargonidin glycosides but contain, in addition to kamferol, glycosides of the 3,4-dihydroxyflavonoids, cyanidin and quercitin. The correspondence of this situation to that described by these same workers for gene *M* in *A. majus* is noted (59). Dodds & Long (60) investigated the inheritance of color in flowers, tubers, and sprouts of cultivated potatoes and found that acylated glycosides of pelargonidin, peonidin, and petunidin are the principal pigments in these tissues. The flowers of *pp R-* plants contain cyanidin (3′,4′-dihydroxy) and peonidin (3′-methoxy-4′-hydroxy) derivatives. In the presence of gene *P*, petunidin (3′,4′-dihydroxy-5′-methoxy) derivatives are found in addition. Tubers of *pp R-* plants contain pelargonidin (4′-hydroxy) and peonidin derivatives while, in the presence of gene *P*, petunidin derivatives occur regularly; pelargonidin is found in the presence of gene *P* only in special cases in which the epistasy of this gene is incomplete. Harborne (61) reports that these pigments of the potato are acylated with *p*-coumaric acid, thus, regardless of their genotype with regard to *P* and *R*, these tissues have the capacity to produce a 4-hydroxy-C_6-C_3 compound. Furthermore, Harborne found that a nonacylated cyanidin glycoside occurs in all four *P* and *R* genotypes (*P- R-*, *pp R-*, *P- rr*, and *pp rr*). Harborne concludes that the biosynthetic path to cyanidin diverges at an early stage from the pathway of the other anthocyanins present. In maize, the gene *Pr* controls the state of hydroxylation of the B-ring of the anthocyanins present in the aleurone of the kernel. *Pr* kernels contain cyanidin (3′,4′-dihydroxy) glycosides while *pr* kernels contain pelargonidin (4′-hydroxy) glycosides [Coe (15)].

Another line of evidence which could support the view that the hydroxylation pattern of the B-ring is established prior to the addition of acetate groups to the C_6(B)-C_3 unit is the occurrence in the same tissue of a variety of flavonoids, all with the same hydroxylation in the B-ring. An excellent example of this is the observation by Geissman, Harborne & Seikel (62) that in *Coreopsis maritima* only 3′,4′-dihydroxyflavonoids are found. These are the chalcones: marein, coreopsin, and butein; the aurones: sulfurein and maritimen; and the 7-glucoside of the flavone luteolin. *Coreopsis gigantea* lacks only the luteolin glucoside. It is particularly interesting that these two species also contain esters of 3,4-dihydroxycinnamic acid (caffeic acid) [Geissman & Harborne (38)].

Analyses of flowers of albino (*-mm-nn*) *A. majus* show that C_6-C_3-C_6 pigments are entirely lacking but that 4-hydroxy- and 3,4-hydroxycinnamic

acids (*p*-coumaric and caffeic acids) are present (38). In view of the nature of the pigments present in nonalbino types, this information could also support the proposal that the hydroxylation pattern of the B-ring is determined prior to formation of the C_6-C_3-C_6 pigment.

The principal arguments against the early determination of the hydroxylation pattern of the B-ring come from two groups of investigations. First, Underhill *et al.* (37) found that, by the dilution standards they used, 3,4-dihydroxycinnamic acid is a less effective precursor of quercitin (3′,4′,3,5,7-pentahydroxyflavonol) than is either cinnamic acid or *p*-hydroxycinnamic acid. This difference could be a consequence of the specificity of a "C_3-modifying" enzyme system. These enzymes might have a greater affinity for 4-hydroxy or unhydroxylated C_6-C_3 compounds than for 3,4-dihydroxy compounds of this class. Second, in *A. majus* the 3′,4′,5,7,-tetrahydroxy-aurone, aureusidin, is present in flowers of plants of the genotype *mm*, in which all other flavonoids bear hydroxyl groups only on the *para*-position of the B-ring (56, 57).

In summary: (*a*) there are some data which appear to be consistent with the view that modifications of the hydroxylation pattern of the B-ring occur after the formation of the C_6-C_3-C_6 nucleus. (*b*) The bulk of the genetic data is consistent with the hypothesis that the hydroxylation pattern of the B-ring is established prior to the use of C_6(B)-C_3 intermediates in the formation of C_6-C_3-C_6 compounds, but this evidence is not unequivocal. (*c*) Finally, mechanisms exist for the hydroxylation, was well as methoxylation of C_6-C_3 compounds at the 3,4, and 5 positions; thus, it is not necessary to postpone these modifications until the C_6-C_3-C_6 compounds are formed. This probably constitutes the best argument in favor of the hypothesis that the hydroxylation pattern of the B-ring is determined early in the course of biosynthesis of the flavonoids. The evidence required to resolve this problem will probably come from enzymological studies.

Modifications of the C_3 segment.—As noted earlier, the flavonoids are divided into classes on the basis of whether or not the C_3 segment is part of a heterocyclic ring and, if so, whether the ring contains five or six members. The state of oxidation of the heterocyclic ring is the final basis for separation. In the preceding section, part of the problem of biosynthetic relations among different flavonoids of the same class was considered. Now the biosynthetic relationships among flavonoids of different classes will be discussed.

These are some of the obvious possibilities: Each class of flavonoid may be derived from a different C_6-C_3 precursor, or a common C_6-C_3-C_6 "key" intermediate may give rise to all classes of flavonoids, each by a different biosynthetic path. The difference between these two alternatives lies in whether the modifications of the C_3 segment, other than participation in formation of the heterocyclic ring, occur prior, or subsequent, to the addition of acetate units to the C_6(B)-C_3 segment and cyclization and aroma-

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tization of the A-ring. The possibility that the flavonoids are derived from one another by oxidations and reductions is implied in the latter alternative. Finally, some parts of the biosynthetic paths may be sequential and subsequent parts parallel, or vice versa. An extensive discussion of the probability of sequential versus parallel synthesis is presented by Seshardi (3).

Underhill *et al.* (37) have proposed a scheme for modifications of the C_3 chain of C_6-C_3 compounds. An adaptation of their scheme, plus some indications of how various classes of flavonoids might arise from different C_6-C_3 compounds, is shown in Figure 3. A number of possibilities are shown for the origin of almost each class of flavonoid, but there is little evidence which can be used to help select the most probable route. The biosynthetic sequence emphasized in this diagram is: (*a*) formation of a key C_6(B)-C_3 unit from shikimic acid; this "key unit" might perhaps be phenylpyruvic acid or *p*-hydroxyphenylpyruvic acid; (*b*) modification of the C_3 side chain by reduction, dehydration, or other reactions; (*c*) modification of the hydroxylation pattern of the B-ring (not shown) by the addition of hydroxyl groups at position 4, or 3 and 4, or at 3, 4, and 5 these steps might as easily occur prior to modification of the C_3 side chain or much later, as discussed above); (*d*) activation of the C_6(B)-C_3 segment; (*e*) addition of three acetate units to the C_6(B)-C_3 segment; (*f*) cyclization and aromatization of the A-ring; (*g*) formation of the heterocyclic ring, except in the chalcones, by either oxidation or dehydration; (*h*) secondary changes in the heterocyclic ring where required to convert one flavonoid into another (e.g., as shown for leucoanthocyanin synthesis); (*i*) and finally (not shown), methoxylation, acylation, or glycosylation of the flavonoid. There are data to support a few of the steps shown in the diagram but most of the reactions shown are speculative.

The biological formation of the heterocyclic ring of the flavonoids has been investigated by Shimokoriyama & Hattori (63) who prepared an enzyme from rays of *Cosmos sulphereus* and *Coreopsis lanceolata* which catalyzes the conversion of hydroxychalcones to hydroxybenzalcoumarononones. The enzyme prepared from a given species is not specific for hydroxychalcones from the same species. Evidence for the course of formation of the six-membered heterocyclic ring, on the other hand, comes only from work in organic chemistry; for example, Geissman & Fukushima (64) have demonstrated the conversion of 2-hydroxychalcones to flavonoids by dilute hydrogen peroxide. Kuroda has demonstrated that carthamin (the 3-glucoside of 2,3,4,6,4′-pentahydroxychalcone) yields glucose and the flavanone carthimidin on treatment with dilute phosphoric acid (65).

The chemical evidence for the conversion of flavanonols to leuco-anthocyanidins and the latter to anthocyanins has been presented earlier in this review. The following observations constitute the principal biological evidence in support of the hypothesis that leuco-anthocyanidins and the anthocyanidins are closely related biosynthetically and may be interconvertible.

Simmonds has found that the white bracts of *Musa acuminata* (banana) contain leuco-cyanidin and leuco-delphinidin but that these compounds are almost entirely absent from mature bracts. Glycosides of peonidin, malvidin, cyanidin, and delphinidin are found in the mature bracts (66, 67). Coe reports that maize kernels of the genetic constitution a_2a_2 *prpr* contain leuco-pelargonidin while A_2-*prpr* kernels contain pelargonidin glycoside; also, a_2a_2 *Pr-* kernels contain leuco-cyanidin while A_2-*Pr-* kernels contain cyanidin glycoside (15). The allele at the A-locus in maize thus appears to to determine whether the anthocyanidin or the corresponding leuco-anthocyanidin will be present in the aleurone layer. Alston & Hagen (14) have shown that the presence of the dominant allele at the *H*-locus in *I. balsamina* L. is perfectly correlated with the occurrence of leuco-pelargonidin in colorless (white) buds and with the occurrence of pelargonidin glycoside in mature flowers. The young leaves of *Cydonia maulei* are red while the larger, older leaves are green; a red-green stage intervenes. Paech & Eberhardt (68) found that the decrease in anthocyanin content which parallels leaf expansion in *C. maulei* is correlated with an increase in both flavone and leuco-anthocyanin. These latter two compounds begin to appear in relatively large amounts after accumulation of anthocyanin in the leaf has stopped but they continue to amass even after all of the anthocyanin has disappeared. Paech & Eberhardt were able to show that a "decolorizing enzyme," which is absent in young leaves of *C. maulei,* is active in older leaves. Macerates of leaf tissue of *Vitis vinifera, Viburnum tomentosum, Parthenocissus tricuspidata, Hedera helix, Fagus silvatica,* and *Crataegus oxyacantha* exhibited "anthocyanin decolorizing activity." The information on decolorization of anthocyanins leads to the interesting, but totally unexplored problem, of catabolism of anthocyanins and the possible function of flavonoids in plant metabolism but this question will not be discussed further here. The data on the appearance of leuco-anthocyanins and flavones concomitant with the disappearance of anthocyanins, plus the observations on the decolorizing system, suggest that interconversions of these compounds may occur in nature. There are a number of possible alternative interpretations of these data and further investigation of this interesting enzyme system may provide valuable information on the metabolism of the anthocyanins. It is particularly important to determine the nature of the product(s) of the "decolorizing" reaction. As for the enzymatic conversion of leuco-anthocyanidins to anthocyanidins, conclusive evidence would be welcome. The anthocyanidins could also arise from some unidentified key C_6-C_3-C_6 compound or, for example, by reduction of a flavonol.

Underhill *et al.* have suggested that phenylenolpyruvic acid (Fig. 3, XXXI) might be the C_6-C_3 precursor of quercitin. Jorgensen & Geissman (69) have suggested the possibility that "the flavonols are produced by pathways quite distinct from those involved in flavone synthesis" as one interpretation of some of their data on flavonoid inheritance in *A. majus.*

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This summary of some evidence—or more correctly, lack of evidence—emphasizes that the diagram in Figure 3 is more an inventory of possibilities in flavonoid biosynthesis than it is a proposal of a particular biosynthetic scheme.

Hydroxylation of the A-ring.—The problem of the modification of the hydroxylation pattern of the A-ring requires attention but this reviewer is unaware of any pertinent information on this matter which has been published since the reviews of Seshardi (3) or Geissman & Hinreiner (1) were prepared.

Light

General.—Light is required for anthocyanin synthesis or may stimulate this process in many species. The older literature is reviewed by Arthur (70) and by Blank (2). Additional instances of an influence of light on flavonoid biosynthesis have been reported recently; in some cases detailed studies of action spectra have been made.

The data of Watkin *et al.* (43) on the incorporation of C^{14}-acetate into quercitin in buckwheat seedlings in light and in darkness show that light is not essential for quercitin synthesis in this species, but a marked stimulatory effect of light on this process is evident. Piringer & Heinze (71) report that light is required for the development of an as yet uncharacterized flavonoid in the cuticle of the fruit of Rutgers tomato. Hillis & Swain found that, on the basis either of percentage of dry weight or of total extractives, more leuco-anthocyanin and "total phenols" accumulate in leaves taken from the sunny south side of a Victoria plum tree (*Prunus domestica* var. Victoria) than in leaves of similar size selected from the shady north side of the same tree. Illumination is also reported to accelerate lignification in the endocarp of the fruit of this species (72). Phillips (73) suggests that the controlling factor in lignification in *Fraxinus excelsior* is illumination of the leaves. Thus, light appears to influence the biosynthesis of C_6-C_3 compounds and of flavonoids besides anthocyanins; a general effect of the level of photosynthetic activity or of accumulated carbohydrates is not excluded in these experiments.

Eberhardt (74) provides another example of the action of light on flavonoid biosynthesis. He found that when five-day-old etiolated red cabbage seedlings were illuminated, the rates of both respiration and anthocyanin synthesis increased. Anthocyanin production continued at a high level during the following two days but the rate of respiration declined sometime between the sixth and seventh days. It was also noted that the respiration of etiolated seedlings of "white cabbage" followed the same course after the commencement of illumination. Eberhardt suggests that the "white seedlings" may produce colorless flavonoids and thus light may have a direct effect on some step in flavonoid synthesis in both red and white varieties. On the other hand, the acceleration of both respiration and antho-

cyanin synthesis could be a manifestation of an increased availability of acetate alone.

Eddy & Mapson (75) found that cress seedlings can make small amounts of anthocyanin in darkness but that illumination with 4 ft.c. of white light greatly stimulates synthesis; 500 to 1000 ft.c. hrs. of white light (incandescent) is saturating for this response. They also found that the effect of light is manifested long after the illumination ceases. The levels of illumination used in these experiments were far below those at which appreciable photosynthesis could be expected. It is not clear from these data whether the light is activating some synthetic system or whether some biosynthetic step is being driven by light energy to produce an intermediate which is subsequently consumed in a nonphotochemical reaction.

The effects of light and other factors on the synthesis of anthocyanin by lemna (*Spirodella oligorrhiza* Kurtz) have been investigated by Thimann & Edmondson (76). They report that a small amount of anthocyanin is formed in darkness by this plant but that more pigment is accumulated when the medium contains sucrose at a concentration of 0.05 *M*. Under constant illumination, at levels of 150 and 800 ft.c., more than twice as much pigment was synthesized in the presence of sucrose as in its absence—thus the effects of light and sucrose appear to be cumulative in lemna. Light is required for the synthesis of idaein (3-galactosidyl cyanidin) by leaf discs of copper beech (*Fagus silvatica* L.) but the synthetic capacity is stimulated by an exogenous supply of sucrose.

Thimann & Radner made the interesting observation that methionine, ethionine, and thiouracil had no effect on the synthesis of anthocyanin in lemna in darkness but had a marked inhibitory effect on the light-mediated synthesis of the pigment (77, 78). For example, plants growing either on water or on 10^{-5} *M* ethionine, were illuminated for three days and were then transferred to fresh medium (water) and darkness. Plants treated with ethionine formed one-half as much or less pigment during the ensuing four days in darkness as did the control plants. It was concluded that

> ethionine must be inhibiting a light reaction whose product is converted to anthocyanin in a dark reaction. The pigment which is formed in dark cultures (not exposed to light) must then either arise from a different and independent reaction, or else, since its quantity is small, it may arise by some method of by-passing the light reaction (78).

Ethionine inhibits the light-stimulated synthesis of anthocyanin in lemna, regardless of whether carbohydrates are provided from photosynthesis or an exogenous supply. Inhibition of anthocyanin synthesis by thiouracil (3×10^{-5} *M*) is reversed by uracil (3×10^{-4} *M*) in illuminated lemna but not in plants maintained in darkness. Copper at a concentration one-tenth that of thiouractil almost completely reverses the effect of the inhibitor. [The anthocyanin of *S. oligorrhiza* Kurtz has recently been reinvestigated by

Geissman & Jurd (79). It appears to be not a cyanin but more probably a pelargonidin derivative. The other flavonoids of this plant have also been examined (80)].

Studies of action spectra.—There appear to be several different patterns of response of anthocyanin synthesis to light quality. Withrow *et al.* (81) have shown that there is a positive correlation between anthocyanin content and irradiance when cotyledons and the hypocotyl of Black Valentine beans are irradiated with light of 725 to 1150 mμ or 710 to 1150 mμ. The wavelengths which are effective in evoking this response are clearly not those effective in photosynthesis. It was also observed that corn seedlings were devoid of any purple coloration when grown in the dark, but the leaf bases became pink when the plants were exposed to red or far-red energy. Klein *et al.* (82) have extended their earlier work on the Black Valentine bean by studying the effects of illumination at 436 mμ and 630 to 700 mμ, as well as light of the wavelength previously studied by them, on elongation of the epicotyl, inhibition of hypocotyl growth, fresh weight of leaf tissue, the synthesis of chlorophylls, and anthocyanin accumulation. They found that 630 to 700 mμ light was most effective both in promoting an alteration in the growth patterns of the tissues and in stimulating anthocyanin accumulation; that light of 436 mμ next most effectively modified all these processes; and that illumination at 710 to 1150 mμ was more effective than at 725 to 1150 mμ. The significant observation here, these workers point out, is that, like the growth responses, anthocyanin production is proportional to the log of the radiant energy supplied. They also observed that, even after illumination for 1 hr. at high irradiances of effective wavelengths, no additional anthocyanin accumulated. A subsequent incubation in darkness (or light) for several hours was required before a significant increase was detectable. Klein *et al.* conclude that the synthesis of anthocyanin in the bean hypocotyl apparently does not involve the direct photochemical conversion of a precursor (as in the case of chlorophyll synthesis) but is coupled to the growth photoreactions (82). This point of view is in direct contradiction to that of Siegelman & Hendricks (83, 84) discussed below.

Piringer & Heinze examined the relation between the accumulation of an unidentified flavonoid in tomato cuticle and light quality. Using filters and the Beltsville spectrograph, they found that the action spectrum for the promotion of pigment development coincides with that for the control of photoperiodic induction of flowering in cocklebur and other species, the germination of seeds of lettuce and *Lepidium,* the expansion of leaves, and other phenomena. They also demonstrated that the effect of irradiation with light of about 600 to 680 mμ, the most effective region of the spectrum for promoting the development of the cuticular pigment, is reversed by radiation of wavelengths of 695 mμ and beyond (far-red). Furthermore, as in the case of the control of lettuce seed germination and other photochemically similar phenomena, the response to a series of red and far-red

treatments depends upon the nature of the last irradiation in the sequence. Piringer & Heinze (71) report that irradiation with far-red light itself elicits some development of cuticular pigment and, consequently, the effect of red radiation is not completely reversed by far-red light. Also, as with some other responses which can be reversed by exposure of the tissues to far-red irradiation, there is a progressive loss of ability of the far-red radiation to completely reverse the effect of the red light with increase in the length of the time between applications of the two wavelengths of light. The synthesis of pigment occurred only in the illuminated area and in the immediately adjacent region (71). [See also Heinze & Borthwick (85)]

Siegelman & Hendricks (84) have investigated the photocontrol of anthocyanin synthesis in the skins of Jonathan, Rome Beauty, and Arkansas apples at wavelengths above 400 mμ. The pigment accumulated is idaein (3-galactosidyl cyanidin). Early harvested apples were stored in sealed polyethylene bags at 0°C. until needed. Then, either whole apples or green areas of skin floated on 0.3 *M* sucrose were illuminated with fluorescent light with a maximum intensity of 1000 ft.c. or with radiation from the Beltsville spectrograph at 13 or 26 joules per sq. cm. throughout the wavelength range studied. During the first 20 hr. of irradiation little or no anthocyanin synthesis occurs or is induced; this "induction period" is slightly longer for the skin of Arkansas apples than for skin from either of the other two varieties examined. The induction period is followed by a "linear period." The amount of anthocyanin synthesized, during the linear period in light and during an ensuing period when light is not required, is a linear function of the time of irradiation at constant irradiance above a threshold of 100 ft.c. during the linear period. The bulk of the anthocyanin synthesized in response to this illumination appears during the dark (nonlight-requiring) period. The extent of accumulation of pigment during the dark period is influenced by the temperature and the duration of this period as well as by the duration of the exposure of the tissues to light during the preceding linear period.

At 15°C., during 48 hr. in darkness, following illumination with the fluorescent source also for 48 hr., the concentration of idaein in pieces of skin from Jonathan apples increased from 1.32 to 2.70×10^{-9} moles per sq. cm. At 25°C. slightly less pigment was synthesized and at 5°C. almost none was produced. No additional anthocyanin was found after 72 hr. in darkness at 15°C. Comparable data on the effect of temperature on the rate of accumulation of pigment during the induction or linear periods are not given for apple skin but are provided from work on anthocyanin formation in turnip seedlings (83). Photoinduction for two hours at 15°C. was effective while at 5°C. no induction occurred in turnip seedlings.

The action spectra for the induction and linear periods are identical. Radiation throughout the visible region of the spectrum is effective in promoting anthocyanin synthesis but a region of maximum effectiveness occurs between about 640 mμ and 670 mμ. A subsidiary maximum appears at about

600 mμ. It is of particular interest that, unlike flavonoid synthesis in tomato cuticles, the effects of visible light are not vitiated by far-red irradiance in the apple skin system.

One clue to the nature of the precursors of anthocyanin in apple skin is provided by an experiment in which the rate of anthocyanin synthesis in apple pieces held in darkness for 20 hr. before irradiation was compared with that of pieces irradiated immediately after preparation. During the dark period, the pieces were floated on 0.3 *M* sucrose solution containing 1×10^{-3} *M* pyruvate. The induction period for the dark-treated pieces was 40 per cent shorter but the rate of pigment synthesis during the linear period was the same in both sets. The important observation was made that during the dark incubation a strong odor of apple volatiles developed which was not evident in the samples irradiated immediately after preparation. Siegelman & Hendricks point out that the volatile products of apples are predominantly acyl compounds and alcohols and suggest that these compounds are either precursors of anthocyanins or are derivatives of such precursors. According to this interpretation, the effect of light is to direct acyl units into flavonoid biosynthesis or to affect the condensation of acetate derivatives with $C_6(B)$-C_3 units. It would be interesting to have data on the accumulation of other flavonoids and of phenols under these circumstances. It might then be possible to determine if the accumulation of apple volatiles is an indirect consequence of a reduction in the availability of $C_6(B)$-C_3 units, either by failure of these units to be synthesized or failure to be activated without light.

The photocontrol of anthocyanin synthesis in turnip seedlings differs from that in apple skin [Siegelman & Hendricks (83)] in two ways: (*a*) the induction period is 2 hr. long, and (*b*) the linear period action spectrum is slightly, but significantly, different. The action spectrum for the promotion of anthocyanin synthesis during the linear period in turnip seedlings shows a low, broad maximum between about 420 and 490 mμ and a sharp, asymmetrical, strong maximum at about 725 mμ. About four times as much pigment is produced by turnip seedlings illuminated with 8 joules per sq. cm. incident energy at about 725 mμ as by seedlings exposed to the same amount of energy in the region of the maximum in the blue. Another action maximum occurs between about 600 and 620 mμ, exposure to light at this wavelength resulting in the production of about one-third to one-half as much pigment per unit energy as when 725 mμ light is used to illuminate the seedlings. The asymmetry of the action spectrum on the short wavelength side of the 725 mμ maximum suggests to Siegelman & Hendricks (83) that "energy is probably transferred in turnip seedlings from the receptor with the indicated maximum at 7250 A to one with a maximum near 6900 A." The strong action maximum at about 725 mμ is reminiscent of the maximum for promotion of anthocyanin synthesis in Black Valentine beans (81).

Like turnip seedlings and some other plant material, red cabbage seedlings require a period of induction in light before the rate of anthocyanin (rubrobrassicin) synthesis becomes proportional to time with constant irradiance. The induction period is only 15 min. in red cabbage seedlings. Anthocyanin synthesis continues for some time in darkness following irradiation during the linear period. The action spectrum for stimulation of anthocyanin synthesis by irradiation during the linear period shows maxima at about 470 to 490 mμ and 690 mμ. Unlike the situation in turnip seedlings, the action maximum in the red region of the spectrum is symmetrical and the response per erg is about the same at the blue as at the red action maximum. The control of anthocyanin synthesis by light in red cabbage seedlings is complicated by the superimposition of the reversible red, far-red "low energy" photomorphogenic system upon the high energy photocontrol system of the linear period. (The "low energy system" is saturated at about 0.1 joules per sq. cm. while fifty times this energy is required to saturate the "high energy system.") Thus, if blue light is used to irradiate red cabbage seedlings during the induction and linear periods, an exposure to red light (about 580 to 690 mμ) is required to obtain maximum pigment production during an ensuing period of either light or darkness. The effect of the red irradiation, as in other cases of this type, is reversed by irradiation with far-red light (about 700 to 780 mμ). These data of Seigelman & Hendricks (83) thus show that the "high energy system" of the red cabbage shares some features with that of the turnip—the difference is the lack, in red cabbage, of the suggested energy transfer mechanism between the two red-absorbing pigments. This is a consequence of the absence of the postulated 725 mμ absorbing pigment in red cabbage. In addition, the expression of the response to effective wavelengths of the high energy system is under the control of the red, far-red reversible "low energy" photomorphogenic system in red cabbage.

Siegelman & Hendricks call attention (*a*) to the similarity of the action spectrum for the high energy response in red cabbage and the absorption spectrum of butyryl coenzyme A dehydrogenase; (*b*) to the accumulation of apple volatiles during incubation of apple skin pieces in darkness; and (*c*) to the involvement of copper in anthocyanin synthesis (discussed below) and the presence of this metal in acyl coenzyme A dehydrogenase. Then they suggest that perhaps the photoreceptor for the high energy system in the 700 mμ region is a flavoprotein, like butyryl coenzyme A dehydrogenase. [Butyryl coenzyme A dehydrogenase catalyses a reaction analogous to Reaction "I" on page 428. The oxidized form of this enzyme has absorption bands at 255, 355, 432.5, and 685 mμ with relative absorbancies of 7.5, 0.87, 1.00, and 0.25 (86)].

Siegelman & Hendricks attribute the maximum found at 600 to 620 mμ in several cases to possible photoactivation of reduced cytochrome-*a*. They propose that during the induction period an acyl II Co A substrate for

anthocyanin synthesis is built up from an acyl I Co A precursor by photoactivation of the oxidized acyl Co A dehydrogenase. Then, presumably during a later part of the induction period, the concentration of the acyl II Co A reaches a high enough level for it to serve as a substrate for the photoactivated acyl Co A dehydrogenase. It is suggested that "the photoactivation process thus is probably being used at least twice in the course of anthocyanin formation" (84). (These steps would correspond to reactions G, H, and I, page 428.) Siegelman & Hendricks (84) add: "This manner of utilization of acyl groups for anthocyanin synthesis is in harmony with the recent observations of Watkin, Underhill & Neish [See reference (43)] on the incorporation of carbon atoms from acetate and sucrose in quercitin by buckwheat."

Before evaluating this proposal further it would be profitable to try to resolve whether the light absorbed by the "high energy system" is used directly in the transformation of precursors in anthocyanin synthesis, as in the proposal just presented, or whether the light effect is indirect via growth responses, as Klein *et al.* (82) suggest from their work with Black Valentine beans. Unfortunately, no quantum efficiency data, which might help resolve this problem, are available.

Mohr (87) studied the action spectra for the control of elongation of hypocotyls of white mustard seedlings and also for the promotion of anthocyanin synthesis in this material. As in Black Valentine beans (82), the action spectra for these two phenomena were identical. Maximal responses in white mustard seedlings, i.e., inhibition of hypocotyl elongation and acceleration of anthocyanin synthesis, were obtained when light of about 710 mμ or about 455 mμ was supplied; there were also indications of possible maxima at several points between 570 and 675 mμ. The observation which is pertinent to the present discussion is that anthocyanin synthesis can be separated from growth responses in these seedlings. Anthocyanin synthesis in the white mustard seedling is under the ultimate control of the "low energy" red, far-red reversible photomorphogenic system. On the other hand, the growth response appears to be under the photocontrol of the "high energy" system alone. Thus, although the full growth response may be manifested, it is not necessarily accompanied by anthocyanin production; and, the growth responses themselves, at least in mustard seedlings, are not sufficient to bring about anthocyanin synthesis. Light-dependent flavonoid formation in tissues like tomato fruit cuticle and apple skin is clearly not accompanied by cell division or enlargement. This serves to show that the light effects are not necessarily indirect via growth responses.

Possible loci of light action.—If it is assumed that light energy (high energy system) is used to drive some reaction which leads to anthocyanin formation, how well does the Siegelman-Hendricks proposal that the light energy is used in the reduction of acyl compounds meet the requirements? Reductions are not required at these points from an organo-chemical point

of view. Indeed, if they did occur, a subsequent oxidation would be necessary to aromatize the A-ring and introduce hydroxyl groups. This certainly does not exclude the possibility that the synthesis does proceed via reductions followed by oxidations, but this course appears to be less economical for the cell than if reductions were omitted at this stage.

What about the possibility that a light-activated reductive step might occur in the synthesis of the $C_6(B)$-C_3 segment? Siegelman & Hendricks (83) suggest this as another possibility. The work of Phillips (73) and of Hillis & Swain (72) showing that lignification and "phenol" accumulation are influenced by light also suggests that some step(s) in C_6-C_3 biosynthesis may be driven photochemically. There are a number of reductive steps in the synthesis of C_6-C_3 compounds from carbohydrates: Kalan & Srinivasan (88) have shown that diphosphopyridine nucleotide stimulates the formation of 5-dehydroshikimic acid from sedoheptulose diphosphate; however, the synthesis of 5-dehydroshikimic acid from D-erythrose-4-phosphate and phosphoenolpyruvate is unaffected by diphosphopyridine nucleotide (34). The triphosphopyridine nucleotide-dependent enzymatic reduction of 5-dehydroshikimic acid to shikimic acid has been reported by Yaniv & Gilvarg (89). The diagram shown in Figure 3 includes a number of places where reductions are required for modification of the C_3 side chain, e.g., the conversion of phenylpyruvic acid to α-hydroxyphenylpropionic acid. Additional information on this aspect of the problem might be obtained by studying the effects of the high light energy system on the metabolism of aromatic amino acids.

The accumulation of apple volatiles by apple skin pieces kept in the dark during the early part of the induction period constitutes the principal evidence which can be interpreted to support the argument that light acts, in anthocyanin biogenesis, to regulate A-ring synthesis rather than the formation of C_6-C_3 units. As discussed earlier, the apple skin data suggest to Siegelman & Hendricks (84) that the acetyl units are directed into flavonoid synthesis to make the A-ring in light, but that in darkness they are diverted for the synthesis of other compounds. But, as pointed out above, this accumulation of apple volatiles could also be interpreted as the consequence of either an inadequate supply of $C_6(B)$-C_3 units to which acetate units can be added or the failure of $C_6(B)$-C_3 units to be activated in darkness.

In every case in which light has been found to affect anthocyanin synthesis, the appearance of the bulk of the pigment occurs hours after illumination of the tissues; in some cases pigment formation can be blocked by "adjusting" the low energy red, far-red reversible system. Thus, it appears that the photochemical step(s) either involve an early precursor of the pigments or that a specific intermediate electron acceptor is reduced in the light and is subsequently oxidized in driving reactions which lead to anthocyanin production. Another possibility is that a synthetic system is activated by light. The role of light in flavonoid biosynthesis is still unclear. The

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current work is reminiscent of the early work on the chemical genetics of anthocyanins in that, generally, the observations have been limited to effects on the concentration of a single class of flavonoids. It may be profitable to examine the effects on structurally related compounds (e.g., aromatic amino acids, phenols, and flavonoids besides anthocyanins) in order to determine the locus of light action. Also, the relations among genetics, flavonoid synthesis, and light have not been explored.

Anthocyanin biosynthesis, complicated as it is, is the simplest known manifestation of control by the low energy red, far-red reversible system. It may offer an extraordinarily suitable situation in which to study this important control system. Some additional aspects of the relation of light to flavonoid synthesis are discussed below.

Pyrimidines and Anthocyanin Synthesis

The inhibition of the light phase of anthocyanin synthesis in lemna by thiouracil and the reversal of the inhibition by uracil has already been discussed. Thimann & Radner expanded these observations by investigating the effects of certain purines and of some pyrimidines other than uracil on the reversal of inhibition by thiouracil (77). Thymine proved to be about as effective as uracil in reversing the inhibition of thiouracil; adenine and hypoxanthine were less effective antagonists of thiouracil action. Besides thiouracil, other purine or pyridimine antagonists, which inhibit anthocyanin synthesis in lemna are, in order of increasing effectiveness: benzimidazole, 2,6-diaminopurine, quinine, azaadenine, and azaguanine. The last-named compound is reported to be 650 times more potent an inhibitor than thiouracil. With the exception of inhibition by benzimidazole, the deleterious effects of these compounds on anthocyanin biosynthesis are partially or wholly reversed by certain purines or pyrimidines.

Thimann & Radner (90) subsequently observed that, in lemna, riboflavin reverses the inhibition of anthocyanin synthesis by ethionine, phenylthiourea, catechol, azaguanine, methionine, sulfadiazine, thiouracil, or quinine. In each of these cases, not only was the inhibition reversed but a stimulation of anthocyanin biosynthesis resulted from the inclusion of riboflavin in the medium. On the other hand, only incomplete reversal of inhibition by sulfanilamide and 2,6-diaminopurine was achieved and inhibition by tyrosine was unaffected by riboflavin. The amount of riboflavin required for reversal of inhibition is reported to be independent of the concentration of the inhibitor. Lemna plants grown on a medium containing 6×10^{-5} M riboflavin made 40 per cent more anthocyanin than did the control plants during a ten-day period. Anthocyanin production is inhibited at higher concentrations of riboflavin. The stimulation by riboflavin and the earlier noted stimulation by sucrose appear to be almost additive; each of these compounds stimulates anthocyanin production in both illuminated and dark-grown plants.

Thimann & Radner also show that the level of endogenous riboflavin is lowered about 50 per cent when plants are grown in the light on a medium containing 3×10^{-6} *M* azaguanine, and that the effect of azaguanine on both anthocyanin synthesis and riboflavin content is reversed when riboflavin (10^{-4} *M*) is included in the medium with the inhibitor. In contrast, azaguanine has no effect on either anthocyanin biogenesis or the level of endogenous riboflavin in plants grown in the dark. It is suggested that the light reaction in anthocyanin synthesis may be primarily the reaction which forms riboflavin. These authors conclude that the formation of anthocyanin "appears to depend upon the increase in riboflavin and not merely on its presence." This conclusion is based, in large part, on the observations that methionine and some other amino acids and azaguanine inhibit while purines stimulate riboflavin synthesis. Purines appear to be utilized for the production of riboflavin (91, 92, 93). The involvement of riboflavin in anthocyanin production seems clear and the parallel effects of azaguanine and methionine on anthocyanin biogenesis in lemna and on riboflavin synthesis are striking. Yet, it is difficult to accept the proposal that anthocyanin synthesis is geared to riboflavin production in the manner suggested, especially in the absence of more information on the rate of turnover of riboflavin in lemna. The need for quantum yield data to help determine the role of light in flavonoid production is again apparent. Thimann & Radner calculate that, for each mole of riboflavin formed, 20 to 65 moles of anthocyanin are produced.

These observations on the involvement of riboflavin in anthocyanin synthesis are particularly interesting in view of the proposal of Siegelman & Hendricks (83, 84) that a flavoprotein may be the photoreceptor in the high energy, light-stimulated systems they have examined. Thimann & Radner show, however, that riboflavin stimulates anthocyanin synthesis in lemna maintained in either light or darkness. Obviously, the action spectrum for riboflavin synthesis must be investigated.

Copper

Edmondson & Thimann report that, at concentrations at which growth is unaffected, phenylthiocarbamide inhibits anthocyanin synthesis in *S. oligorrhiza* Kurtz (94). The inhibition is reversed, at least in part, by the addition of copper to the medium. Two other copper-chelating compounds, salicylaldoxime and diethyldithiocarbamate, are reported to inhibit both growth and anthocyanin synthesis in *S. oligorrhiza*. Siegelman & Hendricks (83) report that J. C. Brown has observed that red cabbage plants grown on copper-deficient muck produce less anthocyanin than do similar plants grown on soils with adequate copper. Edmondson & Thimann found that "anthocyanin extracts" from *S. oligorrhiza* grown in the presence of phenylthiocarbamide differ spectrally from similar extracts prepared from control plants (93). It is not clear whether the principal anthocyanin of these plants

is altered or whether additional pigments are produced under these circumstances.

Respiration, Sugar Feeding, and Anthocyanin Synthesis

A brief summary of work on the relation between "sugar feeding" and anthocyanin synthesis up to about 1949 is given by Thimann & Edmundson (76).

Sugars vary in their capacity to enhance anthocyanin production in *S. oligorrhiza*. Sucrose, at a concentration of 0.015 *M*, promoted anthocyanin synthesis more than did any other sugar tested. Fructose was less effective than glucose; sorbitol was less effective than fructose; galactose and lactose showed little, or no, effect in stimulating anthocyanin synthesis. The addition of glucose or mannitol to the medium resulted in no stimulation of pigment production (76). Thimann, Edmondson & Radner (95) reported that, in general, sucrose promotes pigmentation but has little or no effect on growth, glucose has the opposite effect, and fructose occupies an intermediate position in these respects. Blank (96) found that anthocyanin synthesis in red cabbage seedlings was promoted when solutions containing 0.5 to 1.0 per cent fructose or 2 to 4 per cent glucose or sucrose were supplied to seedlings growing in sand culture; neither of the two latter sugars were effective when supplied at the concentrations at which fructose was effective.

Eddy & Mapson (75) reported that the accumulation of anthocyanin in cress seedlings grown in water culture in darkness can be increased by supplying the seedlings with 1 per cent solutions of glucose, fructose, sucrose, galactose, sorbose, or arabinose. The relation between the stimulation of anthocyanin production and the concentration of glucose in the medium is not linear. One per cent glucose stimulates anthocyanin synthesis to a level about 50 per cent above normal, 2 per cent glucose causes a stimulation of 150 per cent, and 4 per cent glucose of 450 per cent. Eddy & Mapson conclude that anthocyanin production is related more closely to total sugar content of the tissues than to their content of any one sugar. On the contrary, Thimann *et al.* concluded from their studies on lemna that the level of anthocyanin synthesis was positively correlated with the reducing sugar content of the plants until a sugar concentration of about 6×10^{-3} *M* was reached. The anthocyanin content of the tissues showed no correlation with the sucrose content despite the observation that sucrose, of all the sugars fed, was most effective in promoting anthocyanin synthesis. The deficiencies in our knowledge of plant metabolism become very apparent when data of the type just described must be interpreted. The extensive current information on enzymatic reactions in sugar transformations appear to provide little comfort.

Information on the relations among respiration, fat reserves, the respiratory quotient, and anthocyanin synthesis is somewhat easier to interpret.

Paech & Eberhardt (68) found that the rate of anthocyanin synthesis in red cabbage seedlings was greatest during the third to the fifth day after germination of the seed. This is also the time, during the 8-day period following germination which was studied, at which the respiratory rate is highest and the respiratory quotient is lowest. The authors suggests that this is the time at which the consumption of fatty acids is proceeding most rapidly. Eberhardt (74) has shown that the decline in anthocyanin production coincides with the exhaustion of the reserve fats in the red cabbage seedlings. These observations fit well with the demonstrations that acetate is used in the synthesis of the A-ring of the flavonoids, since in the course of degradation of fatty acids acetyl-Coenzyme A is made available, presumably for both anthocyanin synthesis and respiration. It is of particular interest, in connection with the earlier discussion of light effects, that when etiolated seedlings, in which anthocyanin production has begun to decline, are illuminated they start to make more anthocyanin immediately and the changes in respiratory quotient during this phase do not match changes observed during the earlier period. It is suggested that there are some different precursors for anthocyanin synthesis after etiolated seedlings are illuminated. This appears less likely than that the light has a directive effect which facilitates the utilization of the available materials for flavonoid synthesis, perhaps by driving a reaction which goes slowly or not at all without light in older seedlings.

In leaves of *Saxifraga crassifolia* L., the respiratory rate is slightly higher in the red than in the green parts of the leaves but the respiratory quotient is about the same in both tissues. Upon infiltration of leaves with 0.1 *M* sucrose, the rate of respiration is doubled and anthocyanin production is increased tenfold under continuous illumination. Less anthocyanin was synthesized in the same period by infiltrated leaves maintained in darkness [Paech & Eberhardt (68); Eberhardt (74)]. The effects of various inhibitors on both respiration and anthocyanin synthesis in *S. crassifolia* L. were also examined by Eberhardt. Leaves which had been floating on sugar solution were infiltrated with 2×10^{-3} *M* iodoacetate at the time they began to redden. The respiratory rate declined sharply and anthocyanin synthesis was halted. In the presence of 2,4-dinitrophenol the respiratory rate increased while anthocyanin synthesis was arrested; the minimum effective concentration of this inhibitor was 3×10^{-4} *M* but the concentration required to get this response appeared to vary with the age of the leaf. Thimann *et al.* found that 2,4-dinitrophenol inhibited the growth of lemna more than it did pigment formation when the plants were grown on an inorganic medium, but that when sucrose was included in the medium the two processes were about equally sensitive to this inhibitor.

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CYTOCHROMES IN PLANTS[1,2]

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INTRODUCTION

Recently there have appeared several extensive and critical review articles on cytochromes in plants (1, 2, 3). Thus it appears appropriate for this review to attempt only a more detailed evaluation of newer work in several rapidly developing fields and some work that has not been reviewed previously. Emphasis has been placed upon considerations of methodology.

Evidence for possible activities of the cytochrome-linked systems in plants has been seen in: (*a*) oxidative systems in nonphotosynthetic tissues; (*b*) respiratory mechanisms in dark reactions in photosynthetic cells; and (*c*) light induced reactions. This review will cover these different phases of the problem in this order.

CYTOCHROME REACTIONS IN NONPHOTOSYNTHETIC TISSUES

Evidence for the Participation of the Cytochrome System in Respiration

The work on cytochromes in nongreen plant tissues has been directed toward estimating the extent of participation of the "typical" cytochrome system which mediates aerobic oxidations and associated ATP production in yeast and animal tissues. Various approaches to the problem have been made.

The nature of the "terminal oxidase."—A number of oxidases occur in plants, and extensive experimentation has been carried out attempting to show which one actually functions as the so-called "terminal oxidase" in different plant tissues. It is not always clear what is meant by the term "terminal oxidase." Possibly one oxidative pathway may be the one which functions in energy-yielding reactions, while other oxidases have synthetic or other functions. This will be discussed below.

[1] The survey of literature pertaining to this review was concluded in September, 1957. Space limitations have not permitted complete discussion of certain technical aspects of the methods and the results obtained.

[2] The following abbreviations will be used: ADP for adenosine diphosphate; AMP for adenosine monophosphate; ATP for adenosine triphosphate; DNP for dinitrophenol; DPN for diphosphopyridine nucleotide; DPNH for reduced DPN; IAA for indoleacetic acid; P:O is the ratio of ATP formed to oxygen utilized during oxidative phosphorylation; TPN for triphosphopyridine nucleotide; TPNH for reduced TPN; TRIS for trimethylol aminomethane.

Three lines of evidence have cast doubt upon the role of cytochrome-*c* oxidase as the enzyme mediating the main pathway of reaction with oxygen: (*a*) The apparent low affinity for oxygen of the enzyme system mediating respiration in a number of plant tissues; (*b*) the lack of inhibition of respiration by known inhibitors of cytochrome oxidase; (*c*) the apparent lack of cytochrome oxidase activity in homogenates or the presence of only a low order of activity.

Several investigators have now obtained evidence against the first objection listed above. Thimann, Yocum, & Hackett (4) measured the respiration of potato disks in a moist chamber, rather than submerged in water, where the rate of gas diffusion may be limiting; they found a high affinity for oxygen. Yocum & Hackett (5) made similar observations with slices of the Aroid spadix, and Eichenberger & Thimann (6) found the respiration of internode tissue of pea plants to be 50 per cent saturated in a gas mixture containing 3 per cent oxygen. On the other hand Harley, McCready, & Brierley (7) found that the oxygen uptake of excised beech mycorrhizae was dependent upon the oxygen concentration up to 21 per cent oxygen, and this was also true with homogenates of the tissue. It must always be established that oxygen diffusion is not limiting during the measurements. The variable effect of oxygen concentration on cytochrome-*c* oxidase activity in homogenates of leaves and roots observed by Rubin & Ladygina (8) is difficult to explain, particularly since the activity appeared sometimes to go through a maximum with increasing oxygen concentration.

It is well documented that in many plant tissues all or part of the respiration is insensitive to inhibition by cyanide or carbon monoxide. In some animal tissues there has also been observed a cyanide- or carbon-monoxide-resistant fraction of respiration, the mechanism of which is not understood. Hill & Hartree (1) and Hartree (3) have critically discussed all of the difficulties involved in interpreting the evidence from inhibitor studies. It is not always realized that in a reaction mediated by a chain of enzymes, if the inhibitor is not acting near the rate-limiting step, considerable inhibition of the enzyme may not measurably affect the rate of the overall reaction. Also it must be demonstrated that the inhibitor actually penetrates the tissue. For example, azide enters some cells only in the form of the undissociated acid, thus only at rather acid pH. It has been shown that cyanide will react with any keto acids present to form cyanohydrins and it is thus removed.

Articles continue to appear describing the effects of inhibitors on plant respiration, but the data have not yet clarified the situation. Eichenberger & Thimann (6) found the respiration of internode pea tissue to be 80 per cent inhibited by $5 \times 10^{-5}M$ cyanide, and carbon monoxide gave a strong and light-sensitive inhibition. Thimann, Yocum & Hackett (4) had found the respiration of fresh potato disks to be inhibited by carbon monoxide,

with a partition coefficient[3] similar to that of yeast, and the inhibition was light-reversible. However, the inhibitory effect of cyanide and carbon monoxide was lost if the disks were washed for several days or kept in moist air for one day at 25°C. Eliasson & Mathiesen (9) found that the sensitivity of respiration to cyanide and azide varied in different parts of wheat roots, increasing with the distance away from the tip. Kiraly & Farkas (10) observed that the respiration of wheat leaves infected with a rust fungus was strongly inhibited by diethyldithiocarbamate, while the respiration of healthy leaves was not. Thus, they suggested an increase in ascorbic acid oxidase activity in diseased tissues. However, Honda (11) has shown again what confusing results can be obtained with copper enzyme inhibitors such as diethyldithiocarbamate, 8-hydroxyquinoline and phenylthiourea.

The respiration of avocado slices (12) and homogenates of germinating lettuce seeds (13) was found to be unaffected by cyanide although the presence of cytochrome oxidase was demonstrated in each case. Cyanide and carbon monoxide were found sometimes to increase the respiration of slices of Aroid spadix (5), flowers of the spadix (14), and beech mycorrhizae (7). Although there was no inhibition of the respiration of spadix slices with a ratio of carbon monoxide: oxygen of 19, with higher ratios, the K_M for the oxygen uptake was increased (5). From this observation and the partition coefficient[3] of the oxidase for carbon monoxide and oxygen, calculations were made to see whether the lack of inhibition of respiration by carbon monoxide could result from an excess of oxidase activity. It was found that a hundredfold excess of the oxidase over the rate-limiting step would be necessary to account for the data. In the presence of $10^{-3}M$ cyanide 50 per cent of the cytochromes-*a and* -a_3 peak at 605 mμ was oxidized on aeration. Thus, the observations with slices of skunk cabbage spadix suggest that there may be an alternating cyanide-insensitive respiratory pathway in addition to that operating via cytochrome oxidase. The suggestion that this may involve an auto-oxidizable *b*-type cytochrome (3, 15) will be discussed under a later section.

There is disagreement about the effect of inhibitors on the respiration of particulate preparations from plant tissue homogenates, sometimes no inhibition being observed with cyanide and azide; in other instances cyanide was found to produce strong inhibition. For example, although the respiration of the flowers of the spadix of skunk cabbage was not inhibited by

[3] Warburg's partition coefficient for carbon monoxide and oxygen:

$$K = \frac{n}{1-n} \cdot \frac{CO}{O_2}$$

where n = ratio of respiration inhibited by carbon monoxide to the uninhibited respiration, and CO/O_2 = ratio of carbon monoxide to oxygen in the gas phase.

cyanide, that of the mitochondria from the flowers was inhibited by 50 per cent, and the cytochrome oxidase activity of the suspension was completely inhibited by 10^{-4} *M* cyanide (14). The cytochromes-*a* and -*c* of the mitochondria were only 20 per cent oxidized with air in the presence of cyanide. Bonner (16) has found that the azide-sensitivity of the respiration of particles from skunk cabbage spadix can be altered through changes in the pH or the osmotic concentration of the extracting medium. This would appear to be good material to use in studying the relationship of the azide-sensitive and the azide-insensitive respiration.

When Mikhlin & Mutuskin (17) measured the oxygen uptake of homogenates of different parts of barley sprouts in the presence and absence of soluble cytochrome-*c*, they found a variable inhibition by 10^{-2} *M* cyanide. The least inhibition was observed with those tissues showing the greatest increase in respiration on addition of cytochrome-*c*. It was felt that the variability of the effect of cyanide was not due to the presence of keto acids since addition of semicarbazide did not increase the inhibitory effect of cyanide. The authors concluded that the data showed evidence for the participation of other oxidases in respiration. However, the need for caution in interpreting data on cytochrome oxidase assay will be pointed out below. Furthermore, measurements only of the effects of inhibitors on oxygen uptake cannot prove to what extent the different oxidases participate in respiration in the absence of inhibitor.

There is abundant evidence for cytochrome oxidase activity in homogenates of many plant tissues although it has been reported to be lacking from some, and sometimes the activity seems to be of a low order. What is not usually understood is that the assay for cytochrome oxidase is fraught with difficulties; some of these are apparent in recent papers on the subject. Hill & Hartree (1) have pointed out some difficulties in using the manometric method for cytochrome oxidase assay in plant homogenates. We have found that cytochrome-*c* itself (in either the oxidized or reduced form) actually inhibits the oxidase (18). Thus in the presence of concentrations of cytochrome-*c* usually employed in the oxidase assay, the activity is far below the optimum. And if the method is used where the activity is extrapolated to infinite cytochrome-*c* concentration (19), a greatly inhibited enzyme activity is measured. Also recently we have found (20) that another basic protein will strongly inhibit cytochrome oxidase; for example, 10^{-6} *M* salmine will produce about 50 per cent inhibition. These observations may explain some of the difficulties in cytochrome oxidase measurements. Honda (21) found that the cytochrome oxidase activity in homogenates of barley roots increased with the dilution of the suspension as if an inhibitory substance were present. He also found loss of activity if the pH were not kept above 7.0 during the preparation, and the addition of cysteine was necessary to demonstrate the succinoxidase system. Honda later showed (11) that there was enough of either cytochrome oxidase or ascorbic acid oxidase to account for the respiration.

Fritz & Beevers (22) found cytochrome oxidase activity at all stages of development in homogenates of wheat, barley, rice, and oat seedlings. Although the observed rates in homogenates of the roots of wheat and barley were lower than the corresponding respiratory rates, their method of assay (extrapolation to infinite cytochrome-*c* concentration) would have measured rates far below the optimum. They also found loss of activity if the mixture became more acid than pH 7 during grinding.

Simon (23) measured cytochrome oxidase and succinoxidase activities in particles which were probably mitochondria from the Aroid spadix. The cytochrome oxidase activity was increased as much as six fold when the mitochondria were disintegrated by shaking with ballotini beads. Even then the cytochrome oxidase activity was too low to account for the succinoxidase activity, but again this can be explained by the inhibitory effect of cytochrome-*c*. With mammalian mitochondria it has been shown (24) that enzymes do not react readily with cytochrome-*c* in solution unless the mitochondria are first suspended in hypotonic solutions. This might explain the low order of cytochrome oxidase activity observed in some experiments with plant mitochondria (see below).

James (25) states that unfractionated homogenates of some varieties of barley showed no cytochrome oxidase activity although it was present in particles isolated from the homogenates. Others (23, 26) have also found that cytochrome oxidase activity increased when washed particles were prepared from homogenates. This could all fit with the observations of Honda on the effect of dilution of homogenates and with our findings of the inhibition of cytochrome oxidase by basic proteins. The absence of activity in some plant homogenates (27) might also be explained in this way. Johnston, Nasatir, & Stern (28) isolated washed particles from wheat embryos with exceptionally high cytochrome oxidase activity. Eichenberger & Thimann (6) found that homogenates of etiolated pea stems could oxidize ascorbic acid but could oxidize hydroquinone only on addition of cytochrome-*c*, as with mammalian extracts.

One more possible source of error in assaying for the succinoxidase system is apparent from the experiments of Tookey & Balls (29) and Goodwin & Waygood (30) who found the succinoxidase system inactivated by cottonseed phospholipase and by lecithinase in the older seedlings of barley.

In view of the experimental difficulties described, it can be stated that there is so far no evidence that would rule out cytochrome-*c* oxidase as the terminal oxidase in plant tissues. Since the cytochrome-*c* oxidase system is composed of cytochromes-*a* and -a_3, a direct way of demonstrating the presence of this system is by examination of tissues with a spectroscope or a spectrophotometer. This type of observation will be described in the following section.

Observations of absorption spectra of intact tissues.—The classical method for demonstrating the functioning of the cytochrome system in the

respiration of a tissue has involved the observation of the oxidation of the cytochromes (disappearance of bands in the absorption spectrum) on addition of oxygen, and the reduction of the pigments (reappearance of the absorption bands) when the oxygen supply is exhausted. Hill & Hartree (1) have thoroughly reviewed the spectroscopic evidence for the participation of the "typical" cytochrome system in plant respiration. In general in tissues devoid of chlorophyll, a diffuse absorption band can be seen at about 550 to 560 mμ under aerobic conditions, and the usual bands of cytochromes appear when anaerobiosis is established.

Evidence for the presence of absorption bands of cytochromes in non-green plant tissues continues to accumulate. In general the more recent observations add to the previous evidence for the participation of the classical cytochrome system and in addition sometimes the presence of auto-oxidizable cytochromes of type *b*. Martin & Morton (31) saw absorption bands at 530, 552-566 and 600 mμ in beet petiole, Arum spadix, apical stems of onion, and bundles of wheat roots when they soaked the tissues in m*M* cyanide to inhibit the oxidation of the cytochromes. The use of cyanide for this purpose may give misleading results since it will react with any peroxidase present to form a compound with absorption bands around 582 and 542 mμ. Martin & Morton saw no evidence of the cytochrome-*dh* described by Lundegårdh (see below). Several other investigators have also seen absorption bands corresponding to cytochrome pigments in slices of the Aroid spadix (5, 15, 32). In this tissue there appears to be an unusually strong cytochrome-*b* band, a large part of which is auto-oxidizable and, in addition, the usual cytochromes.

Yocum & Hackett (5) were able to measure with a sensitive spectrophotometer the difference in absorption spectrum between anaerobic and aerobic slices of the Aroid spadix and found peaks in the difference spectrum corresponding to cytochromes-*a*, -*b*, and -*c*, as in other tissues where the "typical" cytochrome system mediates the respiration. Most significant is that from the height of the peaks they were able to calculate the concentrations and turnover numbers of the cytochromes in the respiring spadix and found them to be similar to those of other respiring cells (33). In the presence of cyanide they also saw evidence for an auto-oxidizable cytochrome-*b*; this will be discussed later.

Since 1951 Lundegårdh has carried on spectrophotometric studies of the respiratory system of bundles of wheat roots, and the bulk of our knowledge of this respiratory system is based upon his data. Even though Lundegårdh deserves credit for his pioneer work in demonstrating the function of cytochromes in wheat roots, there are certain features of his work on which broad and general conclusions are based concerning root metabolism that should be discussed and critically evaluated. His most recent spectrophotometer mechanically resembles a "slow motion" form of the Process and Instruments recording spectrophotometer (Brooklyn, N.Y.), but the

transmission (rather than the optical density) of the reference material and the sample are plotted separately. In his more recent experiments Lundegårdh balances the turbidity of the wheat root bundles with filter paper in the reference cell. Earlier spectra were recorded at wavelength intervals of 5 mμ (34) and the infrequent data together with other irregularities gave the spectra a jagged appearance that makes the absorption bands of the cytochromes difficult to recognize [(34) p. 485]. Reduction of the wavelength interval to 2.5 mμ (35) resulted in more definitive absorption spectra. With the 17 mm. bundle of wheat roots, Lundegårdh observed changes in optical density as great as 0.2 in the region of the Soret band and thus high sensitivity was not required in his recordings.

The positive results that have been obtained by Lundegårdh on alternating oxygen and nitrogen in the wheat root bundles are that absorption bands of cytochromes-*a*, -*b*, and -*c* appear in less than a minute after aerated medium is replaced by oxygen-free medium. Then the difference spectrum slowly changes from a cytochrome-type spectrum to one showing only a large, broad band. In Figure 1 are reproduced two "crude spectra": absorbancy differences between aerobic roots and filter paper (Curve A) and between anaerobic roots and filter paper (Curve B). The "difference spectrum" is given in Curve C. Lundegårdh [(35), Fig. 5] subjected the curves to a process termed "optical resolution" which is a graphical construction by which the shapes of the absorption bands of known components are successively subtracted from the spectrum of a mixture of components in an arbitrary fashion. Any absorption remaining after subtraction of known components is taken as evidence for unknown cytochromes, such as his postulated cytochrome-*dh*, which has not been seen by other workers. Not only do the original data seem to be of inadequate accuracy for this procedure [(34) p. 450; (35) p. 104], but Lundegårdh could not have had available at the time of some of his publications the necessary absorption spectra of all of the cytochrome components of wheat roots. Thus, many of the spectra he has drawn of the individual components do not represent the experimental data on wheat roots. The recent use by Lundegårdh of chemical reactions that specifically affect different components of the respiratory chain (36) is preferable to his "optical resolution" (37). In contrast to the anaerobic minus aerobic difference spectrum of the wheat roots, which usually consists of a large, broad band (Fig. 1C), unless it is measured immediately after anaerobiosis is established [Lundegårdh (35), Figs. 7, 14, 15], his recent studies with yeast show complete and fairly accurate spectra for the pure components (38) as seen in other experiments (39). Figure 2 shows a typical anaerobic minus aerobic difference spectrum of yeast, with absorption bands of cytochromes-*a* and -a_3, -*b* and -*c* (39).

Another serious experimental difficulty in Lundegårdh's work is that anaerobiosis causes a general, non-specific decrease of absorbancy in the wheat roots (trace B lies below trace A in Figure 1). Wheat roots appear

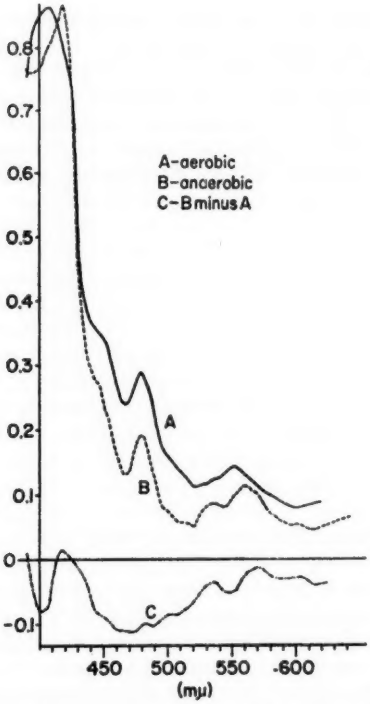

FIG. 1. Lundegårdh's absorption spectra for (A) aerobic wheat roots and (B) anaerobic roots. In each case the blank cuvette contained filter paper. Curve C represents the difference between A and B. (Courtesy *Arkiv för Kemi*)

to be an exceptionally difficult material for spectrophotometric studies.[4] Unfortunately such large changes of opacity on oxidation and reduction would render Lundegårdh's procedure of "optical resolution" even more difficult.

Some of the optical constants obtained for the cytochromes by Lundegårdh are unusual. He found a ratio of γ- to α-bands of only three for cytochrome-*b* (35, 36) for the oxidized minus reduced difference spectrum and a ratio of only two for his postulated cytochrome-*dh* (36). In other systems the ratio found for cytochrome-*b* is about ten (37). Also Lunde-

[4] Difference spectra of yeast (38, 41) ascites, tumor cells, muscle, etc. (37) give satisfactory results, but nonspecific effects have been observed in certain bacteria studied in this laboratory and have been minimized by the use of suitable suspending media, substrates (40), or inhibitors such as azide.

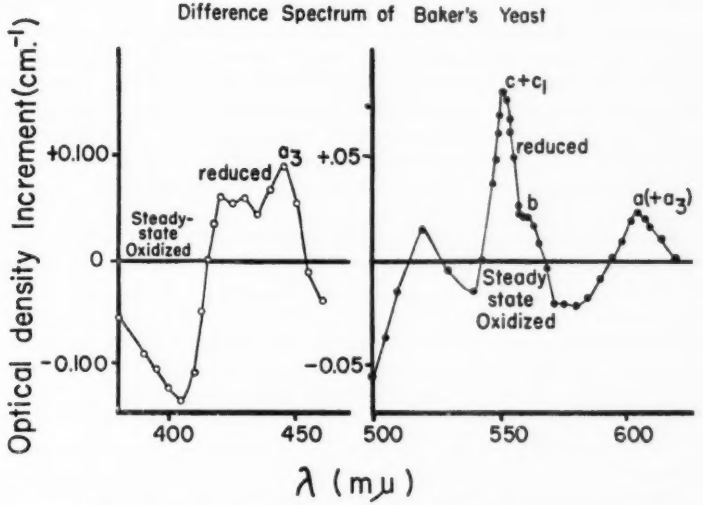

FIG. 2. Difference spectrum of baker's yeast (difference in absorption spectrum between anaerobic and aerobic yeast). The yeast suspension used for measurements in the Soret region of the spectrum (380-460 mμ) was a 4.5-fold dilution of that used for measurements between 500-630 mμ and the left ordinate is multiplied by 4.5. The α-peaks of cytochromes $-a + a_3$, $-b$ and $-c + c_1$ are seen at 605, 562 and 552 mμ. The combined β-bands of the cytochromes $-b$ and $-c + c_1$ are at 525 mμ, and γ-bands of the cytochromes are seen at 445 mμ (cytochrome $-a_3$), 430 mμ (cytochrome $-b$) and 418 mμ (cytochromes $-c + c_1$. [See reference (39)] (Courtesy *Nature*).

gårdh finds the band of cytochrome-*b* in wheat roots to have a much greater width [Lundegårdh (36, Fig. 16)] than that found in yeast (41, 42). Such discrepancies may be due to pigments with broad absorption bands which are not components of the respiratory chain.

Lundegårdh describes a large number of "kinetic" experiments on wheat roots. However, it appears that his measurements of pigment changes reflect the rate of diffusion of reagents. At 19°C. he gives the time for half-maximal oxidation of reduced cytochrome by aerated buffer to lie between 10 to 40 seconds (36). However the actual second order reaction velocity constant for the reaction of cytochrome oxidase with oxygen has been determined with a rapid flow apparatus (43) to be of the order of 10^8 $M^{-1} \times$ sec.$^{-1}$ which would correspond to a half-time of 3×10^{-5} sec. for the concentration of oxygen in aerated buffer. Thus, oxygen diffusion is undoubtedly the rate limiting factor in the measurement of reaction kinetics in the wheat root bundle. The admission of oxygenated buffer probably causes the cytochrome system to pass through a series of 'pseudo' steady

states as the oxygen concentration slowly builds up to its final value. Any interpretation of such data is very difficult and involves the solution of the difficult equations for combined chemical reactions and diffusion; the "kinetic" data do not represent intracellular reaction kinetics as assumed by Lundegårdh [see (36), p. 149]. With an analogue computer we have examined the response of a three component cytochrome chain to oxygen as added in Lundegårdh's experiments, and we find that the sequence of half-times for the appearance of the bands may be the reverse of the chemical sequence. Thus, his general statements based on kinetic studies (44) lack adequate experimental support. Difficulties are also involved in the kinetics of reduction of cytochromes upon exhaustion of oxygen in the roots. Although this is recognized by Lundegårdh (45), reaction sequences are inferred from his data. A useful theorem that has been shown to apply to systems passing through such a sequence of states is as follows: as the oxygen is depleted by respiration, the reduction of the cytochrome components may occur at slightly different rates, but none should reach complete reduction greatly out of step with the others (33). The broad absorption bands which Lundegårdh has observed to appear and to increase in intensity during 30 minutes after cytochromes-*a*, -*c*, and -*b* have been reduced violate this theorem and thus do not represent components of the respiratory chain of wheat roots.

Lundegårdh observed that the appearance of the γ band of cytochrome-*c* on anaerobiosis was not synchronous with the appearance of the α band. He explained this in terms of the selective action of Slater's factor on the γ-band of cytochrome-*c* but with no experimental evidence for this.

Lundegårdh's data suggest that rather low concentrations of oxygen obtain in the tightly packed root bundle bathed in aerobic buffer in the absence of inhibitors. For instance the degree of reduction of the cytochromes is typical of the status of oxygen-starved cytochrome chain, [Lundegårdh (34) Fig. 21; (38); Table 2; (45)]. Also the reduction of cytochrome-a_3 upon addition of O_2-free medium starts with no measurable lag period or "start lag" that Lundegårdh demonstrates for aerobic yeast. Such a "start lag" would be present if the oxygen concentration in the roots were above the saturation value for cytochrome-a_3 at the time of adding the O_2-free medium. Calculations based on the respiratory rate of the roots indicate that flow rates of aerated medium through the root mass must be of the order of one drop per second to have appreciable oxygen left in the effluent (Lundegårdh does not give his flow rates). The possibility that the root bundle, or at least the downstream portion of it, may have been inadequately oxygenated makes it difficult to interpret many of Lundegårdh's experimental results since artifacts could be produced by slight changes in the content of dissolved oxygen in the buffer or slightly different flow rates due to reservoirs of different heights. Also any substances which indirectly affect the flow prop-

erties through the root mass by causing the roots to swell or shrink or which alter the metabolic rate would cause spectroscopic changes in the cytochromes even if there were no direct reaction with the cytochrome system.

The experiments of Lundegårdh on the relationship between anion absorption and the cytochrome system will be discussed in a later section.

Experiments with particulate preparations.—Studies with particulate preparations from broken cell extracts of nongreen plant tissues have tended to show that particles with activities similar to those associated with mitochondria and microsomes from animal tissues can be characterized. Hackett (46) has reviewed much of the experimentation in this field. The cytochrome components associated with the plant particles also have many similarities to those of animal mitochondria and microsomes.

In 1951 Bhagvat & Hill (47) saw the absorption bands of reduced cytochromes appear on addition of substrate to particulate preparations from many kinds of nongreen plant tissues. Rubin & Ladygina (8) reported cytochrome oxidase activity associated with both "plastids" and "cytoplasm" of leaves and roots of barley seedlings. They postulated two forms of the enzyme in the two fractions, but they did not identify the structures in the different fractions. On disintegrating plant tissues, many structures can be broken down into smaller pieces.

Mitochondria: enzymatic activities.—Particles have been isolated by centrifugation of homogenates of different plant tissues at centrifugal forces usually used to separate mitochondria. For example, Hackett (14) prepared spherical granules from the flowers of the spadix of skunk cabbage which were about 1 μ in diameter and were similar to particles seen in sections of the flowers. Numerous experiments with such particulate preparations have demonstrated the presence of the necessary enzymes for electron transport from DPNH, TPNH or succinate to oxygen in potato tubers (48), wheat roots (49), pea epicotyls (50), avocado fruit (51), silver beet petiole (52), lupine seedlings (53), and the sterile tissue and flowers of the Aroid spadix (13, 14, 22, 54). The mere demonstration of the presence of enzyme systems, of course, is not sufficient evidence to draw conclusions about their physiological roles. In some preparations the activity observed was considerably less than the amount expected from measurements of the respiration of the whole tissues; in other experiments there was incomplete recovery when the enzymes in the various fractions were compared with that in the original homogenate. In nearly all of the preparations tested, the cytochrome-*c* oxidase activity was low but possible explanations for this have been considered earlier in this article. The inhibitory effect of Antimycin-A on the succinate- and DPNH-cytochrome-*c* reductase activities was tested in a number of preparations. In lupine mitochondria (53) the oxidation of DPNH and the respiration were completely inhibited by 10^{-8} *M* Antimycin-A, but in other preparations incomplete inhibition was often

observed with concentrations usually inhibitory to the heart muscle respiratory chain. Three comments should be made about the various discrepancies: (*a*) There is an Antimycin-A insensitive DPNH-cytochrome-*c* reductase present in mammalian microsomes; (*b*) Reif (55) observed that some proteins such as serum albumin can bind Antimycin-A and render it ineffective as a respiratory chain inhibitor; and (*c*) the ability of the enzymes of mammalian mitochondria to react with cytochrome-*c* and DPNH in solution is dependent upon the tonicity of the suspending medium since there is a permeability barrier. This problem has not always been considered in experiments with plant mitochondria.

Mitochondria: absorption spectra.—With a visual spectroscope, Martin & Morton (52) saw absorption bands at 553, 563, and 603–4 mμ in wheat root mitochondria treated with hydrosulfite or with succinate plus cyanide. Also when cyanide was added to mitochondria treated with hydrosulfite, a shift in the absorption band at 604 mμ was seen, which they felt was indicative of the reaction of cytochrome-a_3 with cyanide. They pointed out that the absorption band at 553 mμ which would correspond to cytochromes-*c* plus c_1 had a maximum nearer to that of cytochrome-c_1; thus, there may be more of cytochrome-c_1 than of cytochrome-*c*. Difference spectra were measured in a spectrophotometer and also calculated from reflectance spectra, but these do not show good detail. The Soret band corresponding to cytochrome-a_3 cannot be distinguished.

A number of workers have examined the absorption spectrum of mitochondria from the Aroid spadix. In mitochondria from the sterile tissue, Bendall & Hill (15) observed with a visual spectroscope that the absorption bands corresponding to reduced cytochromes were present under anaerobic conditions and disappeared when the suspension was aerobic; the bands also appeared immediately when succinate or malate were added to washed mitochondria. Cytochromes-*a* and -*c* remained reduced when the suspension was aerated in the presence of cyanide, but an auto-oxidizable cytochrome-*b* was also present. It was concluded that the normal cytochrome system was present, but cytochromes-*b* and -*c* were relatively small in amount compared to cytochrome-*a*. The auto-oxidizable cytochrome was called cytochrome-b_7, and it was seen only in the sterile tissue of the spadix. Mitochondria prepared from the flowers of the spadix showed a normal cytochrome spectrum similar to that of yeast. The properties of cytochrome-b_7 will be described in a following section.

Bonner & Yocum (54) saw absorption bands in particles from the spadix of skunk cabbage similar to those seen by Yocum & Hackett (5) in slices. In addition when they observed the spectrum at −196°C., maxima at 550 and 560 mμ could be distinguished. Hackett (56) saw similar absorption bands in mitochondria prepared from flowers of the spadix after clarifying the suspension with deoxycholate. He was able to calculate (57) the difference spectrum (difference between aerobic and anaerobic

mitochondria of spadix) by measuring the two spectra in a Beckman spectrophotometer with a photomultiplier attachment, using filter paper in the blank cuvette. He found peaks in the absorption spectrum at 426–28, 443–45, 480, 525, 553, 558, 562, and 602–5 mμ. From these data he calculated the concentration of functional cytochromes in spadix flowers (those oxidized and reduced) to be around 10^{-6} *M*. In the presence of cyanide the difference spectrum showed the oxidation of cytochromes-*a* and -*c* to be decreased by 80 per cent, but the oxidation of cytochrome-*b* was relatively unaffected. Thus, the difference spectra again show the participation of a "typical" cytochrome system in the respiration and suggest the presence of an auto-oxidizable cytochrome-*b* in the mitochondria from the flowers.

Hackett (57), Lundegårdh (34) and Martin & Morton (52) have all observed absorption bands around 480 mμ in the absorption spectrum of mitochondria or whole tissue. This pigment does not change spectrum when the cytochromes become oxidized and reduced (34).

Microsomes.—Particulate fractions isolated by centrifuging homogenates at centrifugal forces greater than 20,000 g after the "mitochondria" have been removed show some properties similar to those of the microsome fraction of animal tissues.

The "microsomes" from wheat root (49), petiole of silver beet (58), and the Aroid spadix (14, 54) all show Antimycin A-insensitive DPNH cytochrome-*c* reductase activity but little or no succinate cytochrome-*c* reductase activity. In microsomes from silver beet petiole (58) there was an aerobic oxidation of DPNH and TPNH which was not inhibited by cyanide, and this has been suggested as a possibility for a cyanide-insensitive respiratory pathway. However, Hackett (14) found that DPNH and TPNH cytochrome-*c* oxidases in spadix microsomes had five to tenfold less activity than in the mitochondria and suggested that this was too little activity to play a major role in reactions with oxygen.

In the microsome fraction of petiole of silver beet, onion bulbs and Aroid spadix reduced with hydrosulfite, Martin & Morton (58) saw an asymmetric band in the absorption spectrum at about 559 mμ and one at 525 mμ and postulated that these correspond to the cytochrome-b_3 isolated by Hill and Scarisbrick from different plant tissues (absorption bands at 559.5 and 529.3 mμ) since both appeared to be auto-oxidizable and neither combined with carbon monoxide. These absorption bands in the microsomes also appeared on addition of DPNH and TPNH to the anaerobic suspension but not in air. When DPNH was added to wheat root microsomes, absorption bands appeared at 558, 526, 480, and 422 mμ, even under aerobic conditions.

Bendall & Hill (15) saw absorption bands at 562 and 555 mμ in anaerobic preparations of microsomes (or preparations treated with hydrosulfite) from the sterile tissue of the Aroid spadix and also in microsome preparations from pea cotyledons and rat liver. Thus, these observations

are not in agreement with those of Martin & Morton above or with the observations of other workers with liver microsomes (59). Bendall & Hill did not investigate the effect of addition of DPNH to the microsomes, but they concluded from the measurements of redox potential that the absorption band at 555 mμ was not that of cytochrome-*f*.

In some preparations of microsomes (14, 58) a faint band at 605 mμ was also observed. This could result from contamination with particles derived from mitochondria. The relation of this band to cytochrome oxidase activity was not investigated.

Supernatant.—Martin & Morton (52) found that about 61 per cent of the hemin of the original homogenate was in the supernatant fluid. They precipitated proteins from the supernatant fluid remaining after centrifuging down the microsome fraction of beet petiole extracts by dialysis against saturated ammonium sulfate. After addition of hydrosulfite an absorption band was seen at 565–70 mμ which changed to bands at 569–74 and 536 mμ on bubbling with carbon monoxide, suggestive of a peroxidase-CO compound. When another sample was bubbled with oxygen, faint bands appeared at 578 and 540 mμ. Martin & Morton concluded that the bands formed on oxygenation were probably due to a reaction with a denatured protein hemochromogen. However, since oxygenation might also produce hydrogen peroxide, the bands could result from a compound like the peroxidase-hydrogen peroxide compound.

Functions Linked to the Cytochrome System in Plants

Formation of ATP.—Data continue to accumulate indicating that plant particles with properties similar to those of animal mitochondria can, like the latter, carry out oxidative phosphorylation. In animal mitochondria the formation of ATP has been shown to be linked to the respiratory chain reactions (24, 60). The plant systems show similar properties in reactivity, but no observations have been made of absorption spectrum changes in the respiratory pigments of plant particles during phosphorylation.

Particles resembling mitochondria prepared from a variety of plant sources (51, 54, 57, 61 to 64) have been shown to carry out oxidative phosphorylation with P:O ratios as high as 3.8 in the presence of α-ketoglutarate and malonate. Thus, the phosphorylating mechanisms in plant mitochondria can be as efficient as those of animal mitochondria (65). The phosphorylation is "uncoupled" by DNP, and in most cases the respiration was observed to be stimulated on addition of AMP and Mg^{++} ions. In lupine mitochondria (62) AMP was required for the oxidation of α-ketoglutarate. The phosphorylation in sweet potato mitochondria was uncoupled from respiration on addition of catechol in concentrations greater than 10^{-4} *M* unless ascorbic acid was also added (63). The decrease in P:O ratio due to decrease in ascorbic acid was thus suggested as a possible mechanism for senescence. A similar effect of ascorbate in preventing uncoupling in liver

mitochondria by dichlorophenolindophenol has been reported by Siekevitz *et al.* (66). They postulate that the effect of the ascorbate is to keep the dye in the reduced form and that the oxidized and not the reduced form is an uncoupling agent.

In germinating castor bean seedling mitochondria the P:O ratios decreased after five days, and DNP does not stimulate respiration (61). The P:O ratios of mitochondria from cauliflower buds were found to be less than one (67), and there seemed to be present a substance which would decrease the P:O ratios in mitochondria from mung bean seedlings. Thus, there are suggestions of substances that inhibit the phosphorylating process in some plant homogenates.

Growth.—Hackett & Schneiderman (68) showed that the growth of *Avena* coleoptiles in the presence of IAA was inhibited 50 per cent by 2×10^{-4} *M* cyanide and by a ratio of carbon monoxide: oxygen equal to 5. The inhibition of growth by carbon monoxide was reversed by light. On the basis of these data the authors suggest that cytochrome oxidase is of central importance for the functioning of the cells.

Absorption of water.—As with growth of *Avena* coleoptiles, Hackett *et al.* (69) found that the absorption of water by potato disks is strongly inhibited by carbon monoxide, and this inhibition is light-reversible.

The absorption of ions.—The similar effects of many types of inhibitors upon anion transfer and the increased respiration resulting when salts are absorbed by roots or storage tissue suggest a close relationship between ion transfer and electron transfer through a cytochrome chain. This relationship has led Lundegårdh to propose an "electron ladder" hypothesis for anion transport. Since other hypotheses are also being considered, it is important to review critically the experimental basis for this theory which centers about the postulated direct "coenzymatic effect of anions" on the cytochrome system. This often discussed hypothesis of Lundegårdh (44) is proposed as follows:

Cytochrome reduced + anion $>$----------$\rightarrow$ Cytochrome oxidized + e.

Or, representing the oxidized and reduced forms of the cytochrome by Fe^{+++} and Fe^{++}, respectively;

$Fe^{++} + Cl^- >$----------$\rightarrow Fe^{+++} \cdot Cl + e.$

These equations then suggest that the electron can be ejected from the oxidized cytochrome only when an anion (Cl^-, Br^-, NO_3^-, etc.) is present. Lundegårdh states, "at complete absence of movable anions the cytochromes-*a* and -*b* would presumably be fully reduced."

The idea that anions are necessary for cytochrome turnover does not agree with all of the observations that have been made on other hemoprotein enzymes or on the respiratory system of liver mitochondria (60, 70 to 73). In the case of catalase and peroxidase, where the reactions with salts can be accurately studied, it is found that the undissociated acid and

not the anion combines with the hemoprotein (70, 71). Also HCl, HF, et cetera inhibit rather than activate electron transfer reactions in catalase and peroxidase (72, 73). Thus, the anion theory of electron transfer seems to be contrary to the experimental results which show that ligands that bind the sixth coordination place of a hemoprotein interfere with, rather than facilitate, further reactions. Also the specificity requirement for the "coenzymatic" effect is a difficult one to satisfy; F^- inhibits, while Cl^-, Br^- and NO_3^- activate. Phosphate apparently inhibits or may act by a different mechanism (36) which, according to Hagen *et al.* (74a), may involve special sites in the respiratory chain. However, space does not permit any discussion of phosphate transport.

Lundegårdh states that the cytochrome oxidase activity of homogenized wheat roots increases 60 per cent upon addition of 0.1 *M* KCl or KNO_3 (44). However, homogenization of the roots in an anion-absorbing resin did not reduce the oxidase activity to zero as the above equation requires. The possibility that the cytochrome oxidase retained sufficient "native" anions for activity is rendered dubious by the relatively high concentrations of salts used to activate the enzyme compared with those which give spectroscopic effects in the root bundle. Miller & Evans (74) have also tested the effects of addition of salts on the cytochrome-*c* oxidase activity of particles prepared from roots in sucrose containing a low concentration of TRIS buffer. The enzyme activity was found to be stimulated on addition of a number of salts, phosphate being the most effective. Optimal acitvity was observed at a concentration of NaCl of about 0.1 *M*. Also the inhibitory effect of bicarbonate ions on cytochrome oxidase observed by the same authors (75) seems to require too high concentrations of the ion to be of physiological significance particularly since cytochrome oxidase activity is usually present in excess over reductase activities. The activities of most enzymes are influenced by the ionic strength of the medium.

Lundegårdh has made observations on the spectroscopic changes resulting from the addition of salts to the wheat root bundles to attempt to correlate anion respiration with the state of the cytochromes. On addition of ions to roots Lundegårdh has sometimes observed the oxidation of cytochromes-a_3 and -*c* (34) and -*b* (35) and sometimes a reduction of cytochrome-*c* (35), but in many experiments the absorption bands are not clearly defined. In the clearest tracing [Lundegårdh (35, Fig. 22; 44, Fig. 4)] the absorption bands of cytochromes-*a* and -*c* are five mμ too low; thus the wavelength scale was probably in error. Applying the five mμ correction to the absorption peak of cytochrome-*b* would place it at 567 mμ, a value outside of the range for cytochrome-*b* of the respiratory chain. Also the width of the absorption band at 567 mμ is very large (20 mμ). Thus, the most recent results indicate that when the roots are placed in a salt solution, the cytochrome-*a* is oxidized, cytochrome-*c* is reduced or does not change, and an absorption band at 567 mμ, much broader than that of cytochrome-*b*, disappears.

In one experiment (76) kinetic studies of chloride absorption showed a close correlation with spectroscopic changes in the broad "cytochrome-*b*" band. In discussing this time course, Lundegårdh states (35) that "a start period of 10 to 20 minutes is quite adequate for comparison with the present results." By start period, Lundegårdh probably means the time for attainment of the maximum rate of chloride absorption. This agrees fairly well with the slow change of "cytochrome-*b*," which reaches a maximum in about 10 minutes. The change in cytochrome-a_3 was later (35a) found to be half-maximal in 10 sec. and this is too rapid compared with the time course of chloride absorption for a_3 to be involved in the transport process. Likewise the indefinite spectroscopic response of the cytochrome-*c* raises questions concerning the participation of this component. Thus the kinetic evidence favors the participation of the "cytochrome-*b*" in the transport process; but the discrepancy between its kinetics and those of cytochrome-a_3 is so great that this "cytochrome-*b*" component may not be a part of the respiratory chain.

To sum up there appear to be several pieces of experimental data which are inconsistent with the hypothesis that the cytochrome-a_3-*a*-*c* chain is directly involved in anion transport: (*a*) The spectroscopic change in cytochrome-a_3 is too rapid compared with the time course of anion transport. (*b*) The spectroscopic change in cytochrome-a_3 upon addition of salts corresponds to an oxidation, whereas reduction of cytochrome-a_3 would be expected upon increased respiration caused by anion transport. Since oxygen reacts directly with reduced cytochrome-a_3 the basic equation for the respiration rate would be:

$$-\frac{d[O_2]}{dt} = k[O_2][a_3^{++}]$$

It is difficult to find a cause for the increased oxidation of cytochrome-a_3 upon addition of salts, except for the possibility that it is a consequence of increased aeration of the roots, as discussed in an above section.

The addition of anions or of DNP to the roots was found to stimulate respiration, while the addition of anions or DNP gave opposite spectroscopic effects [Lundegårdh (35, Fig. 25)]. On the other hand ATP and anions were found to give similar spectroscopic effects although ATP has not been shown to activate respiration. A very low concentration of ATP (30 μM) caused complete oxidation of cytochrome-*b* (and -*dh*) [Lundegårdh (36, Table 2).] The proposed mechanism for this oxidation is an ATP-driven reversal of electron transfer. If such a reversal can actually be driven by such a low concentration of ATP, it is remarkable that the endogenous ATP does not prevent the reduction of cytochrome-*b* at all times. It should be noted that 3mM ATP caused no measurable spectroscopic effects on liver mitochondria [see also (60)].

The effects of fluoride on the roots show some inconsistencies. Fluoride

does not activate anion transfer but inhibits it instead, although the extra respiration due to transport of other ions is not inhibited (35). The observations that fluoride causes large spectroscopic changes that are attributed to cytochrome-*b* (36) and that the concentration of fluoride used is about the same as that used to demonstrate the presence of peroxidase (77) raises further questions concerning the large "cytochrome-*b*" band.

All of the experiments on spectroscopic changes in roots during anion transport show a lack of evidence for a direct relationship with the "typical" cytochrome chain. Since the response of the broad "cytochrome-*b*" band to the addition of anions correlates most closely with the anion transport, it is possible that this absorption band may be related to a non-respiratory hemoprotein system that is involved in anion transport and this may be of considerable importance. Still, the large effect of fluoride upon the "cytochrome-*b*" band together with the inhibition of anion transport and the lack of inhibition of "anion respiration" need to be explained.

CYTOCHROMES OF TYPE B

As discussed above two characteristics of the absorption spectra of cytochromes in non-green plant tissues are the presence of a broad absorption band at about 560 mμ under aerobic conditions, and also the evidence for the presence in some tissues of auto-oxidizable cytochromes of the "*b*" type.

According to the original designation of Keilin, a cytochrome of type *b*, like that of the typical cytochrome system of yeast and heart muscle, has a hemochromogen-like spectrum with an α-band around 560 mμ. The *b*-cytochromes are more heat-labile than those of type *c*, and they have protohemin as the prosthetic group. The hemochromogen-type linkage can be readily split by treatment with acid or alkali. Some of the *b*-type cytochromes are auto-oxidizable; that is, they can be oxidized in the presence of cyanide.

A number of *b*-type cytochromes have been characterized. The term cytochrome-*b* is used to refer to the pigment in the "typical" cytochrome system. Cytochromes-b_1, -b_2 and -b_4 are pigments found in some microorganisms. Cytochrome b_5 is present in the microsome fraction of mammalian tissues and is part of an active DPNH-cytochrome-*c* reductase. Cytochromes-b_3 and -b_6 are found in plant tissues, the former having a general distribution, the latter being confined to the chloroplasts. An auto-oxidizable cytochrome found in the mitochondrial fraction of the sterile tissue of the spadix of *Arum maculatum* has been called cytochrome-b_7 since it appears to have somewhat different properties from the other cytochromes of type *b*. The properties of plant cytochromes-b_3 and -b_7 will be discussed here.

Cytochrome-b_3.—This term was first applied to a soluble cytochrome isolated by Hill & Scarisbrick (78) from leaf press juice and also from the

colorless parts of plants. They were able to purify the pigment and to measure the absorption spectrum of the reduced compound which showed peaks at 558.6 and 529.3 mμ in concentrated form and an α-peak at 559.7 mμ in very dilute solution. The pigment proved to be auto-oxidizable but did not combine with carbon monoxide. It was easily denatured by alcohol, acetone, and acid.

Martin & Morton (58) prepared "microsome" fractions from a number of nongreen plant tissues and found that on reduction with hydrosulfite the absorption spectra showed bands at 558.6 to 559.5 and 529 mμ. The pigment responsible for the absorption bands was auto-oxidizable and did not combine with carbon monoxide. They concluded that the microsomal pigment was cytochrome-b_3 although the pigment on the microsomes differed markedly from the soluble cytochrome in stability towards organic solvents. As discussed above, plant microsomes possess an Antimycin A-insensitive DPNH-cytochrome-*c* reductase, as do microsomes from mammalian tissues. And in some plant microsome preparations the cytochrome pigment is reduced aerobically in the presence of DPNH, like the cytochrome-b_5 of rat liver microsomes. Martin & Morton (52) found that the ratio of DPNH-cytochrome-*c* reductase activity in the plant microsomes to the cytochrome content was similar to that measured for the cytochrome-b_5 in liver microsomes (79). Thus, the question arises: are cytochromes-b_3 and -b_5 the same pigment, or is cytochrome-b_3 a slightly modified b_5? Since procedures have been described for the purification of both pigments, the experiment which is needed is a comparison of the two purified pigments. Also the question of the identity of the cytochrome-b_3 isolated by Hill & Scarisbrick and the cytochrome in plant microsomes needs similar investigation. Hill & Scarisbrick did not investigate the reactions of their soluble pigment with DPNH, but they found that it was reduced by illuminated chloroplasts in the presence of air. Martin & Morton (58) suggested that this might result from the formation of DPNH on illumination of the chloroplasts. However, there is as yet no evidence for the accumulation of endogenous DPNH (or TPNH) in chloroplasts during illumination. Hill & Scarisbrick suggested (78) that the band seen in the absorption spectrum of aerobic plant tissues might be due to cytochrome-b_3. This fits with the observation that the microsomal pigment can be reduced under aerobic conditions. Hill & Scarisbrick found the pigment to be decreased when the tissue was ground and particles collected by centrifugation at 15,000 g; thus, it could be associated with fractions other than mitochondria.

In a microsomal preparation from the sterile tissue of the spadix of *Arum maculatum* Bendall & Hill (15) saw absorption bands at 562 and 555 mμ when the pigments were reduced by removing air or by addition of hydrosulfite. They cite no evidence for the identity of the pigment with an absorption band in 562 mμ with cytochrome-b_3. They found the same absorption bands in microsome fractions from pea cotyledons and rat liver,

but in this their observations differ from those of the others who have worked with rat liver microsomes, who have seen only the single alpha absorption band corresponding to cytochrome-b_5. Bonner & Yocum (32) felt that the *b*-type cytochrome they found in the mitochondria from skunk cabbage spadix was cytochrome-b_3, although the brief abstract contains very little data. It appears that the pigment here described as cytochrome-b_3 is actually the cytochrome-b_7 discussed below.

Cytochrome-b_7.—The auto-oxidizable *b*-type cytochrome seen by Bendall & Hill (15) in the sterile tissue of the spadix of *Arum maculatum* differs from other cytochromes of type *b* in the following ways: (*a*) it is an auto-oxidizable pigment with visible absorption bands at 560 and 529 mμ in the reduced form; (*b*) it is found associated with the mitochondrial fraction; (*c*) the reduction of this cytochrome is inhibited by urethane, in contrast with the cytochrome-*b* of yeast or heart muscle, and the absorption band of reduced cytochrome-b_7 was seen to disappear completely when the spadix mitochondria were shaken in air in the presence of urethane and cyanide; (*d*) no spectroscopic change is seen on addition of carbon monoxide to reduced cytochrome-b_7; (*e*) titration with the ferro-ferrioxalate system showed the E_0' to be more negative than that of cytochrome-*b*; (*f*) cytochrome-b_7 differs from cytochrome-b_3 in that the latter is readily reduced by both ascorbic acid and leucomethylene blue, while the former is not; thus cytochrome-b_3 has a more positive redox potential.

Hackett (57) also saw evidence for an auto-oxidizable cytochrome-*b* in mitochondria prepared from the flowers of the spadix of skunk cabbage. In slices of the spadix of *Peltandra virginica* Yocum & Hackett (5) also saw evidence for a cytochrome-*b* which could be oxidized in the presence of cyanide.

In the face of so many conflicting conclusions, it is difficult to make a definite decision about the nature of cytochrome-b_3 and -b_7. It would seem best to use the term cytochrome-b_3 for the soluble pigment extractable from all plant tissues and the term cytochrome-b_7 for the auto-oxidizable cytochrome component of spadix mitochondria and to allow considerably more evidence to accumulate about these and the microsomal pigments before final conclusions are drawn.

Possible functions in respiration.—It has sometimes been suggested that the cyanide-insensitive respiration in plants might be mediated by an auto-oxidizable cytochrome-*b* (3). Martin & Morton (58) suggested that the DPNH and TPNH cytochrome-*c* reductases found in the microsome fraction of plant tissues might fulfill this function. However, Yocum & Hackett (5) calculated the turnover number of cytochrome-b_3 from the data of Martin & Morton to be 0.05 per second which is far too slow. The turnover number of cytochrome-b_5 [calculated from the data of Chance & Pappenheimer (79)] is about 0.02. Thus, the experimental data make it unlikely that cytochromes-b_3 or -b_5 participate in cellular respiration.

Bendall & Hill (15) suggested the auto-oxidizable cytochrome-b_7 as a possible alternate pathway to the cytochrome oxidase-cytochrome-*c* system which could be concerned with the cyanide-stable respiration of the Aroid spadix. The respiration of spadix mitochondria may be slightly increased in the presence of cyanide (57). The data on the difference spectra in the presence and absence of cyanide (5, 57) have led to the conclusion that in the absence of inhibitor the normal cytochrome system is functioning, but in the presence of cyanide the oxidation of cytochromes-*a* and -*c* is decreased and then the auto-oxidizable cytochrome-*b* may take over. Unfortunately tests similar to those made with cytochromes-b_3 and -b_5 have not yet been reported for cytochrome-b_7; it is not known whether this component can be oxidized and reduced rapidly enough to account for the rapid respiration rate of the spadix. The cyanide-insensitive respiration shows the same high affinity for oxygen as the respiration in the absence of inhibitor. Even if the cytochrome-b_7 can take over in the presence of cyanide, the question still remains whether the auto-oxidizable systems function at all in the absence of the inhibitor. This is of course a question difficult to test experimentally. But there are two bits of pertinent evidence: (*a*) Bendall & Hill found that the cytochrome-b_7 band of the spadix mitochondria was not completely oxidized in air; (*b*) Hackett observed that the phosphorylating activity of spadix mitochondria decreased in the presence of cyanide. If the assumption is made that only the typical cytochrome-$a + a_3$ respiratory chain is linked to the phosphorylating system, it would seem that this is the only one active in the absence of inhibitor, since P:O ratios as high as 3.8 were obtained in the presence of α-ketoglutarate.

PHOTOSYNTHETIC CELLS

Dark Reactions

Respiration and oxidative phosphorylation.—Evidence has accumulated from a number of sources for the presence of particles resembling mitochondria in homogenates of green leaves. Recently Smillie (80, 81, 82) investigated further the reactions of a fraction of leaf homogenate containing mitochondria, and he demonstrated cytochrome-*c* oxidase activity and a complete Krebs cycle. The fraction was also able to carry out oxidative phosphorylation in the dark with P:O ratios between 2 and 3. The oxygen uptake with α-ketoglutarate was limited by the presence of a phosphate-accepting system. The experimental difficulty in working with mitochrondria from green leaves is that the chloroplasts are easily broken during rupture of the cells, so that the mitochondria are contaminated with chloroplast fragments.

Ohmura (83, 84) also made preparations of particles from leaves which could respire in the presence of a number of plant acids and could fix inorganic phosphate. When the preparation was made with the addition

of ascorbic acid, phosphate could be fixed either in the dark while oxidizing citrate or in the light. From the observations of Smillie and others it appears that in the preparations of Ohmura, the respiration and oxidative phosphorylation were associated with the mitochondria, rather than with the chloroplasts, as suggested by Ohmura. Although Sisakyan & Filippovich (26) claimed to find the greatest cytochrome oxidase in the chloroplast fraction of leaf homogenates, their fractions were not characterized as to their content of mitochondria. They found no activity in the supernatant fraction from centrifugation at 20,000 g, although some chloroplast fragments were present.

Mikhlin & Mutuskin (17) found the cytochrome oxidase activity of leaves to be highest in the rapidly growing segment, and the activity in different parts of the leaf showed a distribution similar to that of chlorophyll and catalase.

It would appear from activity studies that the systems responsible for respiration and the accompanying phosphorylation are associated with mitochondria in green leaves. Since it is not yet possible to get the mitochondria free of chloroplast fragments, spectrophotometric studies with the mitochondria would be difficult (see below).

Difference spectra.—When the difference in absorption spectrum between anaerobic and aerobic green leaves or green algae (*Chlorella* or *Chlamydomonas*) is measured (85, 86), there is observed so much change in absorption spectrum due to noncytochrome pigments that the difference spectrum of the cytochrome system is obscured. However, with a pale green mutant of *Chlamydomonas,* which has relatively low concentrations of chlorophyll and carotenoids, the anaerobic minus aerobic difference spectrum appears to be that typical of cytochromes-*c* and -*b* with scarcely detectable amounts of cytochromes-*a* plus a_3. In the anaerobic minus aerobic difference spectrum of the mutant there was also an absorption peak corresponding to DPNH and a trough characteristic of flavoprotein.

The dark difference spectrum of the photosynthetic bacterium, *Rhodospirillum rubrum,* shows an absorption peak typical of cytochromes in the Soret region of the spectrum (400-450 mμ), but in the visible region (450-650 mμ) there are bands due to pigments other than cytochromes (87).

Light Induced Reactions

As mentioned above, two cytochromes have been found to be associated with the chloroplasts of green parts of plants: cytochromes-*f* and -b_6. And Vernon, Elsden, Kamen, & Newton (88, 89, 90, 91) have isolated in good yield two kinds of cytochromes from photosynthetic bacteria. One kind has an absorption spectrum similar to that of cytochrome-*c,* but the redox potential is nearer to that of cytochrome-*f*.

Oxidation of cytochromes.—Kamen & Vernon (92, 93) have observed that reduced mammalian cytochrome-*c* (or the *c*-type cytochrome of the bacteria) can be oxidized when illuminated in the presence of aerobic extracts of the photosynthetic bacteria. They postulated a special "cytochrome-*c* photo-oxidase" in these bacteria. However, since the oxidation requires the presence of oxygen, it could be of no importance in the light reactions of the photosynthetic bacteria which grow anaerobically in the light and evolve no oxygen. Also it has been shown (94) that the system in *Rhodospirillum rubrum* which mediates this oxidation is heat stable and thus may not be an enzyme system. Nieman & Vennesland (95) have observed a similar oxidation of reduced cytochrome-*c* on illumination in the presence of a digitonin extract of chloroplasts and oxygen. The system in this case was shown to be heat sensitive. To assess the physiological importance of such a system, it should be demonstrated that high enough oxygen concentrations are present to satisfy the oxygen requirement of the system. If the cytochrome-*c* of green leaves is on the mitochondria, rather than the chloroplasts, it is difficult to see how the reaction can take place. The spectrophotometric observations to be reported below show no evidence for the oxidation of cytochrome-*c* on illumination of aerobic cells of a pale green mutant of *Chlamydomonas.*

Krall (96) has observed the inhibition of carbon dioxide fixation by pretreatment of leaves with a mixture of 95 per cent carbon monoxide —5 per cent oxygen. The inhibition was decreased by illumination with yellow light, and the yellow light was more effective than red light. He also found (97) that exposure to carbon monoxide decreased the incorporation of P^{32} by illuminated barley leaves; the level of labeled ATP was increased by illumination with yellow light. Krall postulated the participation of a cytochrome oxidase in the photosynthetic carbon dioxide fixation and in the esterification of ATP. As pointed out by Gaffron (98) it is not possible to tell whether the inhibitory effect of carbon monoxide is a direct one on photosynthesis. The relief of carbon monoxide inhibition of carbon dioxide fixation by yellow light required as long as five minutes which may be too long a time for photodissociation. Also, Whatley, *et al.* (99) have demonstrated light induced formation of ATP in chloroplasts in the absence of oxygen.

Hill (100) has stated that he and Scarisbrick could find no oxidase specific for cytochrome-*f* which could catalyse its oxidation by molecular oxygen. Thus, it has been inferred (101) that cytochrome-*f* (and probably cytochrome-b_6 also) functions in photosynthesis, in the reactions leading to the formation of ATP during illumination. But a correlation between changes in the steady-state of cytochromes and the rate of photosynthetic phosphorylation such as has been found for the oxidative phosphorylation system (60) is lacking at present.

Study of Photosynthetic Cells with Physical Methods

The effect of illumination upon photosynthetic organisms has been studied by a variety of physical methods. Three sensitive techniques that have given results are electron resonance absorption, fluorescence measurements and absorption spectrophotometry.

Electron resonance absorption.—This method will detect free radicals with high sensitivity. Sogo *et al.* (102) and Commoner *et al.* (103) have reported evidence for the formation of free radicals upon illumination of *Chlorella*. The identity of the substance(s) involved is not indicated by this method, but some aspects of its reaction kinetics suggest that it may be related to a compound with a band in the absorption spectrum around 518 mμ (see below). From Commoner's observations it seems unlikely that changes in pyridine nucleotide or cytochromes are involved.

Fluorescence measurements.—Changes in fluorescence during illumination would be expected if pyridine nucleotide were reduced. Duysens' experiments with purple bacteria (104) showed such changes in fluorescence. His observations are not in agreement with the direct spectrophotometric measurements of absorption spectrum changes in *R. rubrum* at 340 mμ on illumination (88). Strehler & Lynch (105) and Duysens & Sweep (104) could obtain no clear evidence for the accumulation of reduced pyridine nucleotides during illumination of *Chlorella* by fluorescence measurements. This method is not applicable to the study of cytochromes.

Absorption spectrophotometry.—Since the cytochromes are characterized by their bands in the absorption spectrum, it would seem logical to use this method to demonstrate their participation in light induced reactions in the photosynthetic cell. Although numerous measurements of this kind have been made, the experiments and the interpretation of the data have not always been made with the necessary precautions. The remainder of this review will be devoted to a consideration of such spectrophotometric studies.

Apparatus for absorption spectrophotometry.—Two general types of spectrophotometric apparatus are used in studies of the response of photosynthetic cells to actinic illumination[5]; one kind records continuously, the other intermittently. The second type operates only in the absence of actinic light but is suitable for measurements of spectrophotometric changes resulting from high intensity flashes of actinic light. This method has been used in the experiments of Kok, Lundegårdh and Strehler & Lynch. The method which records continuously during illumination operates more satisfactorily at lower levels of actinic light. The apparatus for continuous recording can involve either a "compensating beam" method, as used by Duysens and in some experiments of Strehler & Lynch or a "double beam" or two monochromator method, such as that used in the investigations in this laboratory.

[5] By actinic illumination, we mean illumination, usually with red light, sufficient to activate photosynthetic reactions.

With the continuous recording method it is possible to follow a complete cycle of metabolic changes, beginning with the dark anaerobic cells and following their transition into photosynthetic activity on illumination, then back again to the initial conditions. On the other hand, with the intermittent method, repetitive flashing of the actinic light gives a steady state which may be considerably different, depending upon the lifetimes of the dark intermediates. Witt's data (106, 107) show that the dark reaction is very rapid and that the unavoidable delay in the response of the intermittent methods should be specified. Kok (108) measured the change in absorbance of cells for an interval of four milliseconds and compared it with the value of 60 milliseconds later. It is clear from Witt's data that the absorbance is changing rapidly in the interval of Kok's first measurement and that a change in the rate of the dark reaction from fast to slow at different wavelengths would cause the slow changes to be recorded at relatively greater magnitude than the fast ones. Such a possible artifact could only be controlled by recordings with a continuous type of instrument. The capacitance-coupled amplifier usually used with the intermittent method is unresponsive to slow changes of light absorption and only shows the rapid changes. The continuous methods record slow changes as well as fast and must be rendered insensitive to slow, nonspecific absorbance changes, such as those resulting from the settling of the cells. The "compensating beam" method does not rule out such changes since only the measuring beam passes through the cell suspension, and the base line is not entirely stable in recording changes in *Chlorella* suspensions [Duysens (109, Fig. 3 & 4)]. The "double beam" instrument does compensate to a considerable extent for such nonspecific changes, provided the two monochromators are set at wavelengths 10 to 20 mμ apart.

Although Latimer (109a) and Keilin & Hartree (109b) have reported errors in the location of the absortion maxima or in the absorbancy values for absolute spectra of scattering material, the favorable photocell-cuvette arrangement, together with the procedure of measuring difference spectra, make these errors negligible in studies with the double beam apparatus.

Possible sources of error and necessary controls in absorption spectrophotometry.—Besides instrumental problems described above, several other experimental difficulties in spectrophotometric studies of absorbancy charges resulting from actinic illumination should be considered.

(*a*) An inconsistency is apparent in the use of light absorption to measure the effects of illumination, and precautions must be observed to insure that there is no interference of the actinic and measuring beams. Control of the effect of the measuring light upon the cells may be obtained in at least two ways: (*i*) no photosynthesis should be detectable when the cells are exposed to the measuring light and (*ii*) a decrease in the intensity of the measuring beam should not cause an increase in the absorbancy change caused by the actinic beam.

(*b*) Although the possible effect of the actinic illumination in producing

changes in selective scattering (110) or in fluorescence yield (111, 112) have been discussed recently, these effects would appear to be small or negligible.

(*c*) In assessing the participation of the cytochromes in the illumination reactions, knowledge of the known absorption spectra of the cytochromes must be applied in interpretation of the difference spectra obtained. The instruments used in measuring the difference spectra are sufficiently accurate to expect that the position of the peaks in the difference spectra would agree closely with those in the difference spectra of the isolated cytochromes. Agreement to within $\pm$ 1 mμ could be expected in measurements at room temperature and to within 0.3 mμ when the spectra are measured at liquid air temperature. Also there should be correspondence between the heights of the α, β and Soret peaks to within about $\pm$ 20 per cent of the known relative amplitudes of the peaks. The difference spectra between reduced and oxidized cytochromes-*c* and -*f* are plotted in Figure 3. More definitive results could be obtained if the formation of chemical derivatives of the pigments with known absorption spectra were measured. Unfortunately this is difficult with cytochromes-*c* and-*f* because of their lack of reactivity.

(*d*) In making spectrophotometric studies of cell suspensions, it is important to establish that the absorbancy change recorded coincides with the activity under investigation. A convenient method for measuring photosynthesis in green cells is the measurement of oxygen evolution by means of the platinum microelectrode. Many misunderstandings could be avoided by proper monitoring of the physiological response.

Summary of the experimental results from absorption spectrophotometry.—When the changes in light absorption of photosynthetic cells upon actinic illumination are plotted as a function of wavelength, "difference spectra" are obtained, as described above. In considering all of the data of this type which has accumulated, two things are apparent:

(*a*) The "actinic difference spectra" of green cells and purple bacteria exhibit absorption bands that are clearly not those of cytochrome pigments, and these confuse the identification of the cytochromes. In describing the three absorption bands seen in the difference spectra of *R. rubrum* in both dark and light, Chance & Smith (87) commented, "the peak around 515 mμ is large and that at 428 mμ is small in relation to the peak at 550–563 mμ" compared with typical cytochrome difference spectra, and they suggested that pigments other than cytochromes might be contributing to the difference spectra. In green leaves and algae a large and broad absorption band is seen in the difference spectra around 518 mμ, the nature of which is not yet definitely established, although a change of a carotenoid has been suggested to explain one phase of the absorbancy change (86).

Duysens (113) observed that when *R. rubrum* suspended in aerobic distilled water were illuminated, an absorption band appeared at about

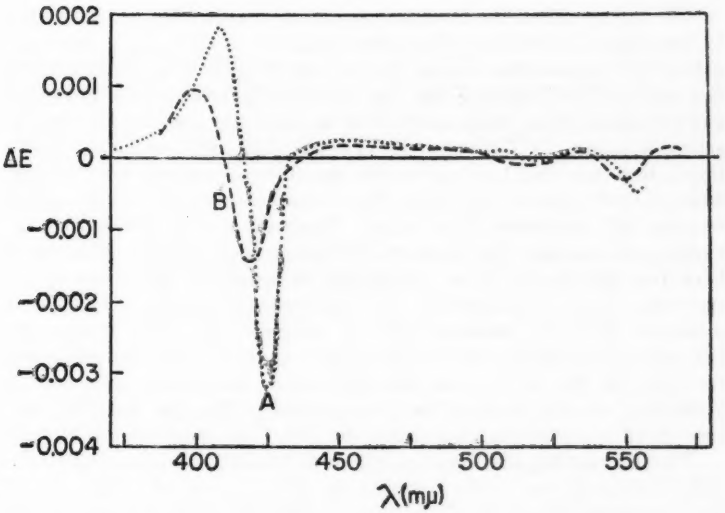

FIG. 3. The difference in absorption spectrum between oxidized and reduced cytochrome-*c* (A) and cytochrome-*f* (B). Replotted from Hartree (3). (Courtesy Interscience Publishers).

432 mμ which was much broader than that characteristic of cytochrome pigments. He suggested that the absorption band might be related to the oxidation of bacteriochlorophyll and that this is only seen when the cytochrome pigments are not reduced. Similar changes in absorption spectrum were also observed (87) when *R. rubrum* were illuminated in the presence of phenylmercuric acetate which inhibits the reduction of cytochromes in these bacteria.

(*b*) When the data are reviewed in relation to the possible errors in methods pointed out above, the evidence for the experimental demonstration of cytochrome changes during illumination is meager.

There have been a number of reports of evidence for changes in cytochrome-*f* on illumination. Strehler & Lynch (105) obtained a different spectrum in *Chlorella* plotted from the slow "negative overshoot" of the dark reaction, rather than from the initial phase of the reaction resulting from turning off the actinic light. They assume that the difference spectrum is the mirror image of the fast dark reaction. The fact that the cytochrome-*f* in their spectrum showed the opposite type of an oxidation-reduction change from that observed in the continuous recording apparatus suggests that this assumption might not have been valid at the wavelengths in the region of cytochrome-*f*.

The time required for Lundegårdh to make a measurement (114) after he illuminated *Chlorella* was that necessary for the transfer from the illumination tube to an optical cuvette plus at least 10 seconds to record the first few points of the difference spectrum and about 30 seconds to complete the measurements. These times are so long as compared with the kinetics observed by others for *Chlorella* [for example, Witt (106) and Kok (108)], that it is certain that Lundegårdh was measuring a different phenomenon. More recently Chance and Sager have obtained clear recordings of the response of cytochrome-*f* to actinic illumination in a *Chlamydomonas* mutant and conclude that no more than 10 per cent of their effect could have remained by the time Lundegårdh recorded the first point of his spectrum. Thus, it is possible that Lundegårdh's results were from a persistent effect of photosynthesis. A possible explanation is that the *Chlorella* Lundegårdh used for his "dark" spectrum were anaerobic and the *Chlorella* that he observed following actinic illumination were aerobic because of oxygen produced in photosynthesis. Thus, his spectrum may correspond to an aerobic minus anaerobic difference spectrum in *Chlorella.*

The fact that Duysens found a peak in the actinic difference spectrum of *Chlorella* at 420 mμ (115) suggests the participation of cytochrome *c,* not cytochrome-*f* (see Fig. 3), [Duysens (115), Fig. 1; and Hartree (3)]. But in the red alga *Porphyridium cruentum,* he recorded (116) an α-band at the wavelength corresponding to cytochrome-*f,* while the γ-band was at the wavelength of cytochrome-*c.* Kok's spectra (108) show a Soret band at the correct wavelength for cytochrome-*f* but the corresponding α-band is nearly merged with a very large peak at 565 mμ. If the latter is the result of reaction of cytochrome-b_6, as he suggests, a large Soret band (10 times that of the alpha peak) should also be apparent, and it is absent. In Spruit's experiments (117), freezing and thawing of *Chlorella* seemed to cause the disappearance of the α-band but not the γ-band of cytochrome-*f* in the actinic difference spectrum. This evidence raises the question whether the α-band can be attributed to a cytochrome in these experiments. Chance & Strehler (85) did not find definitive evidence for cytochrome-*f* in the actinic difference spectra of *Chlorella* and *Chlamydomonas.* But Chance & Sager (86) were able to see prominent α- and γ-bands of cytochrome-*f* at the proper positions and relative intensities in the difference spectrum of a photosynthetically active pale-green mutant of *Chlamydomonas* which is deficient in chlorophyll and carotenoids (Fig. 4). The cytochrome-*f* peaks were not apparent in the dark anaerobic minus aerobic difference spectrum of the mutant. In the red algae the oxidation of cytochrome-*c,* or cytochrome-*f,* or both, probably occurred on illumination. In neither the pale-green mutant nor the red algae was there an appearance of the band at 518 mμ on illumination (86).

In summing up the various observations, it appears that there has been no incontrovertible demonstration of changes in the absorption spectrum

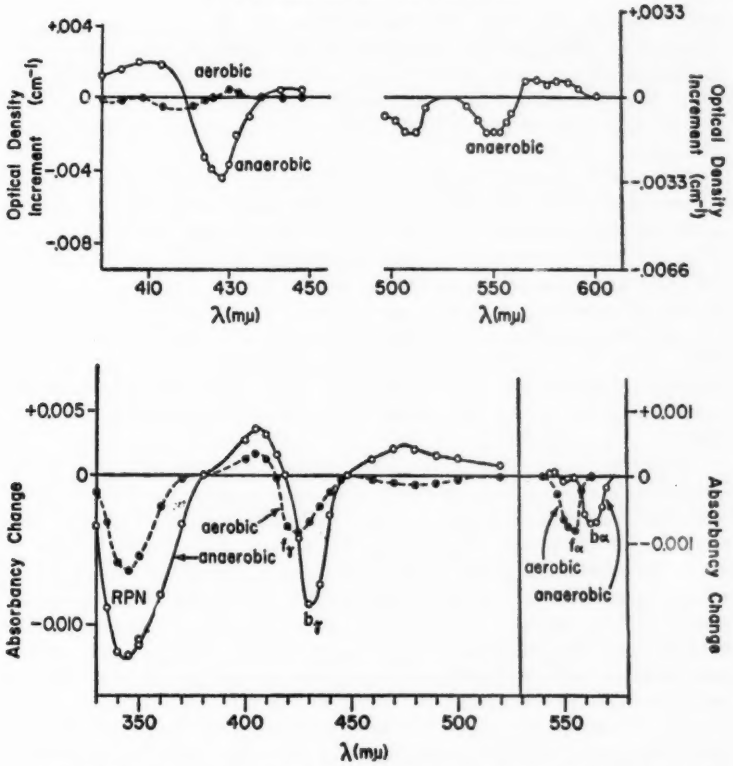

FIG. 4. Upper curve: the difference in absorption spectrum between dark and illuminated anaerobic *Rhodospirillum rubrum*. In the Soret region of the spectrum (400–450 mμ), an absorption peak disappears on illumination which would be characteristic of the oxidation of a cytochrome of type *b*. The absorption peaks in the visible region of the spectrum are not similar to those of cytochrome pigments. The data are replotted from Chance & Smith (87). (Courtesy *Nature*).

Lower curve: difference in absorption spectrum between dark and illuminated cells of the *Chlamydomonas* pale green mutant in both aerobic and anaerobic states. Illumination of the aerobic cells causes the disappearance of absorption bands corresponding to reduced cytochrome-*f* and would thus represent the oxidation of cytochrome-*f*. The data are replotted from Chance & Sager (86). (Courtesy *Plant Physiology*).

of cytochrome-*f* during illumination of green cells with a usual complement of chlorophyll and carotenoids. This might be due to the masking of changes in cytochrome-*f* by changes in absorption spectra of other pig-

ments. So far, the only clear-cut evidence for the oxidation of cytochrome-*f* on illumination is that seen in the *Chlamydomonas* mutant low in chlorophyll and carotenoids. The possibility is not ruled out that in cells with usual concentrations of chlorophyll and carotenoids there is not a measurable change in the oxidation-reduction state of cytochrome-*f* during illumination.

The *Chlamydomonas* mutant shows distinctive absorbancy changes in the region of 340 mμ that are attributed to the oxidation of reduced pyridine nucleotide upon illumination of the aerobic or anaerobic cell. This appears to be a clearer result than Duysens' recordings of absorbancy changes in *Chlorella* attributed to the reduction of pyridine nucleotide (115). Also, the two attempts to demonstrate pyridine nucleotide reduction in *Chlorella* by measurement of fluorescence changes have not given positive results (104, 104a). Thus, the reduction of pyridine nucleotide added to isolated spinach chloroplasts has not yet been observed to occur upon illumination of the intact green cell. A full discussion of this discrepancy appears elsewhere (86).

Although observations with normal green cells have not given easily interpretable results, some pertinent data have resulted from experiments with the *Chlamydomonas* mutant and with *R. rubrum*. With these two kinds of cells the following observations have been made: Illumination of anaerobic cells causes an oxidation of cytochrome pigments similar to those oxidized on aeration. This oxidation could be the result of reaction with some oxidizing equivalents formed on the photolysis of water or, in the case of the *Chlamydomonas* mutant, from oxygen evolved. Or, in either case, it might arise indirectly by some other mechanism. In both kinds of cells the pigment predominantly affected seems to be a cytochrome of type-*b*, that is, a cytochrome with a Soret peak in the region of 430 mμ. In addition, cytochrome-*f* is also oxidized on illumination of the *Chlamydomonas* mutant, and cytochrome-c_2 in *R. rubrum* (118). When the anaerobic *Chlamydomonas* mutant is illuminated, the change in the cytochrome-*f* band is not affected as rapidly as that of cytochrome-*b*, the lag in response of cytochrome-*f* being as long as 20 seconds.

In *Chromatium* which is an obligately anaerobic photosynthetic bacterium, Olson (119) has shown changes in absorption spectrum when aerobic cells are illuminated. But the results are complicated by the observation that components of the respiratory chain are further oxidized under these conditions. In the presence of the appropriate concentration of phenylmercuric acetate, which inhibits the reduction of the cytochromes in these bacteria, illumination of aerobic *Chromatium* resulted in the disappearance of bands in the absorption spectrum at 421.5 and 555 mμ suggesting the oxidation of a cytochrome of type *c*. Such a cytochrome might act similarly to cytochrome-*f* in green cells.

In conclusion there are some data which appear to show specific oxi-

dation-reduction changes of cytochrome during illumination, but these are clear-cut only in certain types of photosynthetic cells. Also absorption spectrum changes of cytochromes similar to those seen on reaction with oxygen are apparent on illumination. Perhaps the most interesting of all the observations is that the absorption spectrum changes not related to respiratory enzymes seen on illumination of green cells or photosynthetic bacteria are also seen during reaction with oxygen in the dark (84a, 85, 87).

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